Human metapneumovirus: Investigation of epidemiology strain diversity and human immune response

Thesis

Human metapneumovirus: Investigation of epidemiology strain diversity and human immune response.

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I confirm that the work presented in this thesis has not previously been submitted for a degree or any other qualification to this or any other university or institution. The work presented in this thesis is entirely my own, except where gifts or contributions from colleagues are fully acknowledged

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Abstract

Human metapneumovirus (hMPV) is a newly described respiratory virus belonging to the *Paramyxoviridae* family, first identified in respiratory samples of children with acute respiratory tract infection (ARTI). Since its discovery hMPV has been associated with ARTI worldwide, however, important questions remain as to the contribution of hMPV to respiratory illness, and its impact on public health.

Extensive surveillance of hMPV within different populations of the United Kingdom (UK) demonstrates it is an important cause of ARTI in the elderly, and influenza like illness (ILI) in people of all ages in the community. Furthermore it is a frequent cause of hospitalisation in young children.

Recombinant baculovirus expressed hMPV nucleocapsid protein (N) proved to be a useful source of antigen for the development of an hMPV specific ELISA. Analysis of age stratified sera from the UK indicates that a majority of children are infected by the age of 6 years with primary infections occurring throughout infancy. Virtually all adults have detectable levels of hMPV antibody; however, reinfection with hMPV is common, raising questions concerning acquisition and duration of immunity.

Sequence analysis of the attachment glycoprotein (G), shows a high degree of nucleotide and amino acid variation and extensive glycosylation. Frequent nucleotide insertions or deletions result in frame shift mutations which can drastically alter the appearance of the protein and often results in premature termination. Antibody recognition of hMPV G is likely to be highly strain specific and dependent on the extent of glycosylation, suggesting an involvement of G in immune evasion.

Phylogenetic analysis of hMPV G gene sequence shows that whilst a large degree of variation exists within this gene, strains circulating within the UK are genetically similar to strains circulating elsewhere in the world, and similar strains circulate throughout different years and populations within the UK.

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III. Abbreviations

Ab	Antibody
AcMNV	Autographia californica nuclear polyhedrosis virus
Ag	Antigen
AGM	African green monkey
AMV RT	Avian myeloblastosis virus reverse transcriptase
APV	Avian pneumovirus
ARTI	Acute respiratory tract infection
BAL	Bronchial Alveolar Lavage
BSA	Bovine serum albumin
cDNA	Complimentary deoxyribonucleic acid
CDV	Canine distemper virus
CMV	Cytomegalovirus
COPD	Cardiopulmonary disease
CoV	Coronavirus
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocytes
dN	Non synonymous
DNA	Deoxyribonucleic acid
dS	Synonymous
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
EV	Enteroviruses
F	Fusion protein
G	Attachment glycoprotein
G(s)	Attachment glycoprotein secreted
GAG	Glycosaminoglycans
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GE	Gene end
G-FL	Attachment glycoprotein full length
GP	General Practitioner
GS	Gene start
GSP	Gene specific primers
HeV	Hendra virus
HIV	Human immunodeficiency virus
hMPV	Human metapneumovirus
HMW	High molecular weight
HPA	Health protection agency
HRP	Horse radish peroxidise
hRSV	Human respiratory syncytial virus
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILI	Influenza like illness
IVIG	Intravenous immunoglobulin
L	Polymerase

LLC-MK2	Lewis ling carcinoma monkey kidney 2 cell line
LMW	Low molecular weight
LRTI	Lower respiratory tract infection
M	Matrix protein
M2	Matrix 2
MAb	Monoclonal antibody
MeV	Measles virus
MMLV RT	Moloney murine leukaemia virus reverse transcriptase
MOI	Moiety of infection
mRNA	Messenger ribonucleic acid
MuV	Mumps virus
MW	Molecular weight
N	Nucleocapsid protein
NASBA	Nucleic acid sequence based amplification
NDV	Newcastle disease virus
N-FL	Nucleocapsid full length
Ni-NTA	Nickel -nitro acetic acid
NiV	Nipah virus
NK	Natural killer cells
NPA	Nasopharyngeal aspirate
NS	Non-structural gene
OD	Optical density
ORF	Open reading frame
P	Phosphoprotein
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PCR	Polymerase chain reaction
PFU	Plaque forming units
Pi	Post infection
PIV	Parainfluenza virus
PVM	Pneumovirus of mice
RCGP	Royal college of general practitioners
RD	Respiratory distress
RMK	Rhesus monkey kidney cells
RNA	Ribonucleic acid
RNAi	Interfering ribonucleic acid
RNP	Ribonucleoprotein
RSV	Respiratory syncytial virus
RT	Reverse transcription
RTI	Respiratory tract infection
RT-PCR	Reverse transcription - polymerase chain reaction
RV	Rhinovirus
SARS	Severe acute respiratory syndrome
SeV	Sendai virus
SF9	Spodoptera frugiperda cells
SH	Small hydrophobic protein
siRNA	Small interfering ribonucleic acids
SV40	Simian virus 40
SV5	Simian virus 5
TNF	Tumour necrosis factor
TS	Transition

TV	Transversion
UK	United kingdom
URTI	Upper respiratory tract infection
USA	United states of America
vRNA	Viral ribonucleic acid
WB	Western blot

Chapter 1

General Introduction

1.1. Newly identified or emerging viruses

Over the past 30 years the number of newly emerging, re-emerging or identified viral pathogens has risen considerably in both humans and animals. Viral pathogens are of a particular concern to human and animal health because of their enormous contribution to burden of disease, the limited availability of antiviral treatments, their rapid adaptability to environmental change, acquisition of antiviral resistance, and the potential for zoonotic transmission.

The underlying causes for the emergence of new viruses are due to generalized social changes (urbanization, IV drug use and sexual practices), ecosystem disturbance (increased human mobility, civil unrest, deforestation, and hunting), economic and commercial trends (intensive farming, farming practices, global trade, and exotic meat trade), medical interventions (organ transplant, blood transfusion, antibiotics and antivirals) and climatic change (global warming) (Kuiken et al., 2003a). As a result many viral pathogens, for example; HIV; SARS coronavirus; Ebola; Marburg; West Nile virus and Avian Influenza, have expanded their host range or ability to transmit to new species.

Other new viruses however, have arisen, or rather; have been discovered as a result of improved diagnostic efforts in the search for previously unidentified etiological agents or in the hunt for host reservoirs of newly emerging diseases. These new viruses are therefore often classed as newly identified (having been present in the host species for a number of years) rather than newly emerging (human coronavirus NL63(van der Hoek et al., 2004), human metapneumovirus (hMPV)(van den Hoogen et al., 2001), and human coronavirus HKU1(Woo et al., 2005)). The most recent of which is human bocavirus (hBoV) a newly identified parvovirus associated with LRTI (Allander et al., 2005).

Both newly emerging and newly identified viruses are of great importance and investigations into these new agents are vital for evaluating their potential threat to a species or population, their contribution to burden of disease, and potential benefit of antiviral or vaccine development.

1.2. Acute respiratory tract infections

Acute respiratory tract infections (ARTI) in humans are one of the leading causes of morbidity and mortality worldwide, and as such have enormous economical implications

(Monto, 2002). A wide range of viruses, bacteria and fungi are associated with respiratory illness, and therefore an accurate diagnosis of infection is vital for correct patient management, and the implementation of preventative strategies such as vaccination. Despite this however more than 50% of patients with ARTI each year are not assigned a cause (Louie et al., 2005, Zambon et al., 2001). This may be attributed to diagnostic limitations, lack of recognition by existing detection methodologies and by still unknown pathogens.

A large number of viral pathogens from a number of virus families have been associated with respiratory illness including the *Adenoviridae, Coronaviridae, Orthomyxoviridae, Paramyxoviridae* and *Picornaviridae* families (Table 1.1).

Table 1.1 Characteristics of selected human respiratory viruses.

Virus family	Viruses	Genome	Virion Structure
Adenoviridae	Adenovirus [a]	Double stranded DNA 30-42Kb	Non- enveloped Icosahedral capsid
Coronaviridae	NL63, 229E, OC43, SARS	Single stranded positive sense RNA 27-32Kb	Enveloped Helical capsid
Orthomyxoviridae	Influenza A[b], B and C	Segmented negative sense RNA 12-15 Kb	Enveloped Helical capsid
Paramyxoviridae	Parainfluenza virus [c] Respiratory syncytial virus [d]	Single stranded negative sense RNA 13-16Kb	Enveloped Helical capsid
Picornaviridae	Human rhinoviruses [e] Enteroviruses [f]	Single stranded positive sense RNA 7.2-8.4 Kb	Non-enveloped Icosahedral capsid

Footnotes: (a): At least 51 serotypes are known. (b): Divided into further subtypes. (c): 4 subtypes known. (d): 2 Subtypes known. (e): More than 100 serotypes are known. (f): More than 71 serotypes are known

Many of the newly emerging or newly identified viruses over the past 30 years belong to these families and have been associated with respiratory tract infections (RTI) in humans.

The focuses of this thesis is on the recently identified respiratory pathogen, human metapneumovirus (hMPV). At the start of this research very little was known about hMPV but over the past 5 years there has been a great deal of work on this virus. This introduction gives an overview of Human metapneumovirus and the different aspects of work that have been reported since its discovery.

1.3. The newly identified human metapneumovirus (hMPV)

In 2001 human metapneumovirus (hMPV) was identified by van den Hoogen et al in the Netherlands (van den Hoogen et al., 2001). HMPV was detected in nasopharyngeal aspirates taken from 28 epidemiologically unrelated children over a 20 year period presenting with RTI using a combination of cell culture isolation, electron microscopy, and random polymerase chain reaction (PCR) techniques. HMPV has since been reported worldwide. Although newly identified, serological evidence of hMPV infections dating back to 1958 has been reported indicating hMPV has been present in the human population for at least the last 50 years (van den Hoogen et al., 2001), viral isolation over the past 10 to 20 years has also been reported in Europe and North America (Boivin et al., 2002, van den Hoogen et al., 2001) .

HMPV has been associated with ARTI in people of all ages, but is especially prevalent in young children, where 1-25% of respiratory tract infections have been associated with hMPV. Seroloprevalence studies indicate that virtually all children have been infected by the age of 5- 10 years (Ebihara et al., 2003, van den Hoogen et al., 2001, Wolf et al., 2003). The detection of hMPV in people of all ages suggests that despite universal infection in childhood repeated infections occur throughout life. This is likely to be due to incompletely protective immune responses and virus diversity (Peret et al., 2002).

HMPV has a seasonal distribution typical of a majority of respiratory viruses. In temperate countries most cases are reported during the winter and spring months, whilst in sub-tropical countries peak activity occurs in the spring and early summer. Phylogenetic analysis shows that there are two lineages of hMPV; A and B, each with at least 2 sub-lineages (A1, A2 and B1, B2) (Bastien et al., 2003, Mackay et al., 2004). It has recently been reported that an additional A sub-lineage (A3) may exist (Huck et al., 2006). The two subtypes of hMPV have been shown to co-circulate at the same time in the same geographical locations. As yet there is no evidence to suggest that these subtypes are associated with differences in severity of disease. A more detailed discussion regarding hMPV epidemiology is presented in chapter 3 of this thesis.

Based on sequence homology and gene order hMPV was placed as the first human member of the *metapneumovirus* genus of the subfamily *Pneumovirinae,* of the family *Paramyxoviridae* as illustrated in Figure 1.

1.4. The Paramyxoviridae Family

The *Paramyxoviridae* contain some of the most infectious viruses known for both humans and animals such as measles, mumps, distemper, Newcastle disease, parainfluenza (PIV), and respiratory syncytial virus (RSV), Paramyxovirus infections are therefore extremely costly in terms of burden of disease in both animals and humans and subsequent economic impact. Over the last decade several new members of the family in addition to hMPV have been identified mostly in animals such as snakes (Franke et al., 2001), fish (Kvellestad et al., 2003), shrews (Tidona et al., 1999), and pigs(Philbey et al., 1998). Others such as the Nipha and Hendra viruses have been found in both animals and humans and shown to cause serious respiratory and neurological illness (Chua et al., 1999, Murray et al., 1995).

The *Paramyxiviridae* belong to the order of *Monoegaviriales* along with the *Filovoviridae*, which include the Marburg and Ebola like viruses, *Rhabdoviridae* which includes the lassaviruses such as rabies, and *Bornaviridae* whose sole member is the borna disease virus. The *Paramyxiviridae* are divided in to 2 subfamilies the *Paramyxovirinae*, which contains 5 genera, and the *Pneumovirinae*, which contains 2 genera (Figure 1.1) (Büchen-Osmond, 2001 onwards). The classification of these viruses is based on morphological criteria, genome organisation, biological activities of the viral proteins, and sequence relationships.

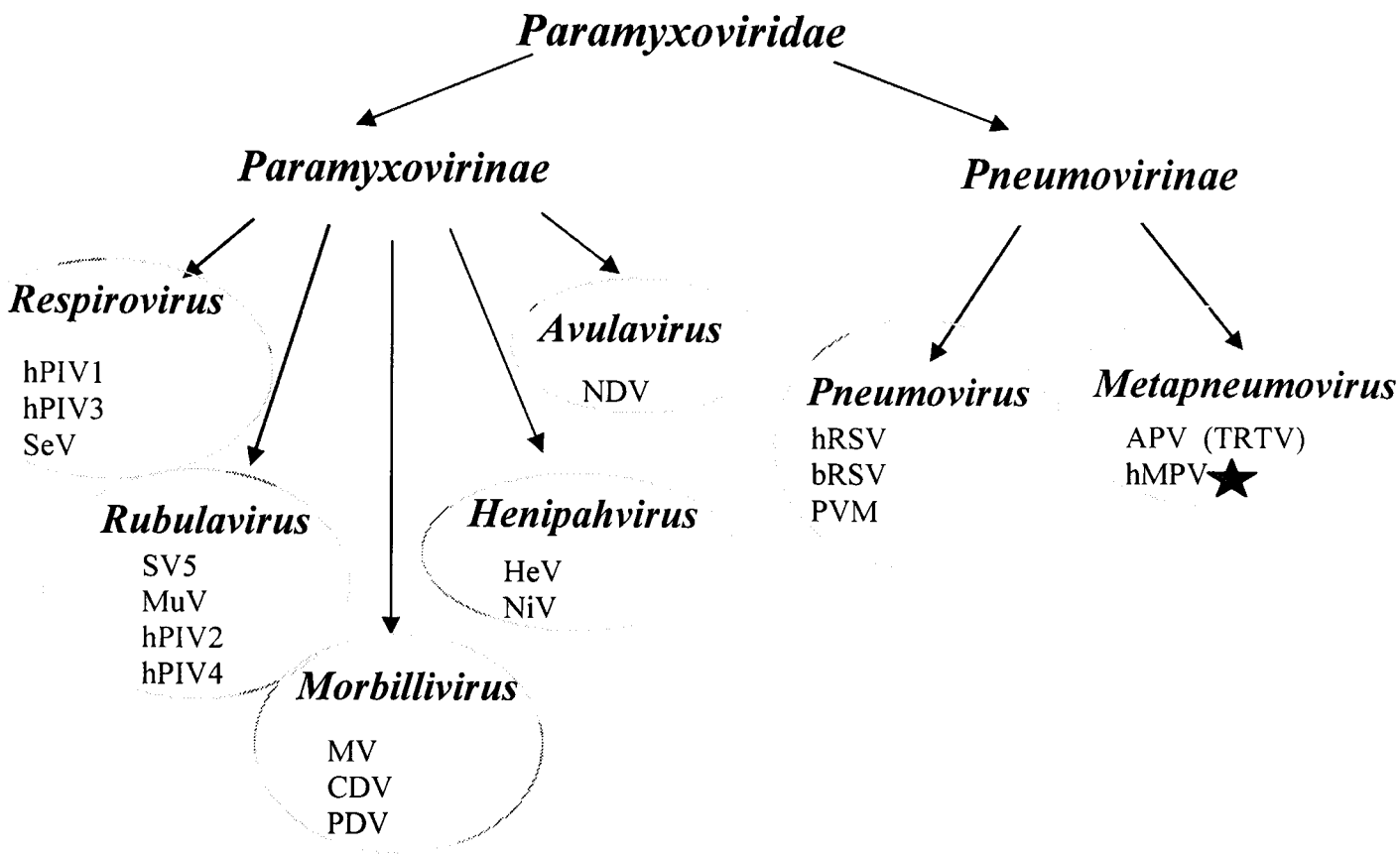


Figure 1.1: Schematic representation of *Paramyxoviridae* taxonomy. Abbreviations: hPIV: human parainfluenza, SeV: Sendai virus, SV5: Simian virus 5, MuV Mumps virus, CDV: Canine distemper virus; PDV porcine distemper virus; HeV: Hendra virus; NiV: Nipah virus; NDV: Newcastle disease virus; RSV: respiratory syncytial virus; PVM: Pneumovirus of mice; APV: Avian Pneumovirus; TRTV: Turkey Rhinotracheitis virus hMPV: Human Metapneumovirus (Büchen-Osmond, 2001 onwards).

1.4.1. **The** *Pneumovirinae* **Subfamily**

The *Pneumovirinae* subfamily contains two genera, the *Pneumoviruses* for which human respiratory syncytial virus (hRSV) is the prototype and the *Metapneumovirus* for which avian pneumovirus (APV) is the prototype, which are described below. Where the term Human *Pneumovirinae* is used it refers to hMPV and hRSV.

1.4.2. Avian Pneumovirus (APV)

APV is the closest genetic relative to hMPV and is the only other member of the metapneumovirus genera. APV is the cause of severe respiratory infection in turkeys and was first identified in South Africa in the 1970s (Buys & Preez, 1980), but has since been found world wide in a range of birds such as chicken, ducks (Shin et al., 2002, Toquin et al., 1999), ostrich (Cadman et al., 1994), geese and pheasant (Gough et al., 1988). To date there are 4 subtypes of APV (A to D). Subtype C is most closely related to hMPV(van den

Hoogen et al., 2001). APV C outbreaks occur mainly in the spring and autumn and the spread of disease to domestic poultry has been associated with migratory birds (Shin et al., 2000). For industry this disease has devastating consequences and as such has been the focus of much research on the detection, prevention and treatment of the disease (Cook, 2000, Cook & Cavanagh, 2002, Njenga et al., 2003). The close genetic relationship of hMPV and APV C suggests that hMPV may have appeared in the human population as a result of zoonosis. Juvenile turkeys experimentally infected with hMPV however did not support virus replication. Together with data showing serological evidence of hMPV in the human population for at least the past 60 years this suggests any zoonotic event that may have occurred will have happened over 60 years ago (van den Hoogen et al., 2001).

1.4.3. Human Respiratory Syncytial Virus (hRSV)

hRSV belongs to the pneumovirus genus and is the closest human virus relative to hMPV (van den Hoogen et al., 2001). hRSV was first isolated in 1956 from a symptomatic chimpanzee (Blount et al., 1956), and shortly after from infants with respiratory disease(Chanock et al., 1957). hRSV is prevalent world wide and is now known to be the most important cause of lower respiratory tract infection (LRTI) in young children with 90% having been infected by the age of 2 years (reviewed by (Collins et al., 2001). There are 2 groups of hRSV A, and B, which co-circulate primarily during the winter and spring months. Some reports suggest that viruses belonging to different groups differ in pathogenicity, however other reports have contradicted this (Hornsleth et al., 1998, McIntosh et al., 1993, Walsh et al., 1997). Reinfection with homologous and heterologous strains of hRSV occurs frequently throughout a person's lifetime but are usually limited to the upper respiratory tract. However hRSV may cause pneumonia in elderly and immunocompromised individuals (Collins et al., 2001).

1.5. Pneumovirinae structure

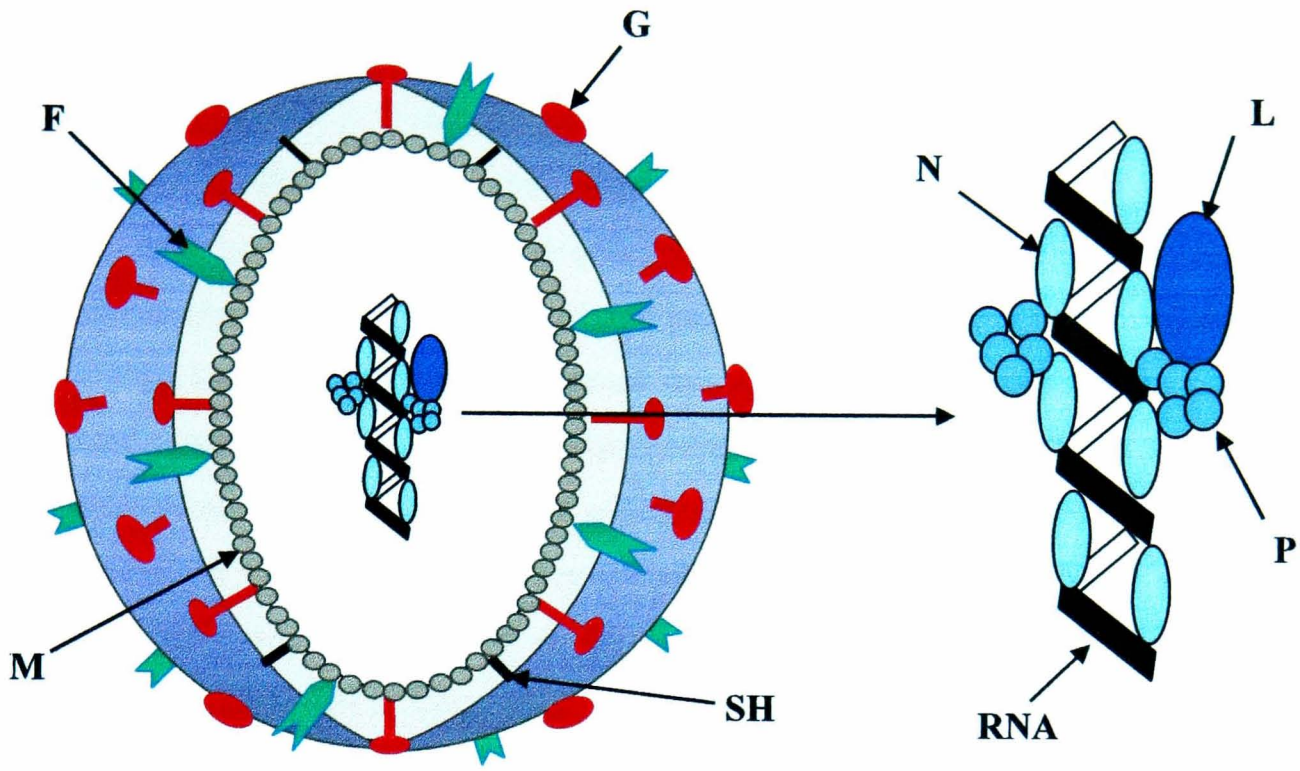


Figure 1.2 Schematic of the Pneumovirus particle adapted from (Easton et al., 2004). The Proteins of the RNP complex (N, P, L and M2-1) associate with the genomic RNA to form a helical structure. THE RNP complex is surrounded by the matrix protein and a lipid envelope in to which the viral glycoproteins G, F and SH are inserted.

Visualisation of *Pneumovirinae* virions by electron microscopy (EM) shows virus particles that have an irregular spherical or filamentous shape of approximately 150 to 300nm in size. Virions consist of a nucleocapsid core displaying helical symmetry. The core of the virion consists of the RNA genome which is associated with the nucleocapsid (N) protein, phosphoprotein (P), polymerase (L), and M2-1 proteins which form the ribonucleoprotein (RNP) complex. A lipid bi-layer envelope derived from the host cell plasma membrane surrounds the virions. Inserted into the envelope are 3 virally encoded transmembrane glycoproteins; the attachment glycoprotein (G), the fusion protein (F), and the small hydrophobic protein (SH) which are organised on the envelope surface and appear as short surface projections of approximately 11-20nm in length by EM. Residing between the RNP complex and the lipid envelope is the viral matrix protein (M) which interacts with both the RNP complex and the internal regions of the surface glycoproteins (Collins et al., 2001).

1.6. Genome structure and organisation

The hMPV genome consists of a single negative strand of RNA approximately 13kb in length. It contains 8 genes, which encode for 9 proteins, based on homology with hRSV and APV. From 3' to 5' these are the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), M2 which encodes for the transcription elongation factor (M2-1), and the RNA synthesis regulatory factor (M2-2), small hydrophobic protein (SH), attachment glycoprotein (G), and the large polymerase protein (L) (van den Hoogen et al., 2001). The 3' and 5' ends of the genome encode regulatory sequences termed the leader and trailer sequences. The 3' leader sequence in the genome sense RNA directs replication and transcription, while the 5' end trailer sequence (3' in anti genome sense) directs replication. At the start of each transcribable gene there is a gene start signal (GS), at which transcription initiates, and at the end a gene end sequence (GE), at which point transcription terminates (Collins et al., 2001, Easton et al., 2004)

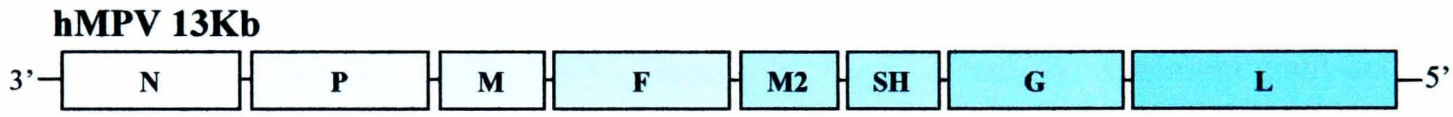


Figure 1.3 Schematic of hMPV and hRSV genome organisation.

There are a number of notable differences between the genomes of hMPV and hRSV, which separate them in to their different genera. The first is that hRSV contains two non-structural genes; NS-1 and NS-2 at the 3' end of the genome. These genes are thought to play a role in antagonising host antiviral interferon responses (Bossert & Conzelmann, 2002, Spann et al., 2005), and the lack of these in hMPV may be associated with the typically milder course of illness and the difficulties in isolating hMPV in cell culture. The gene order also differs between the two genera. As shown in Figure 1.3 the F and M2 genes of hMPV are located closer to the 3' end of the hMPV genome than in hRSV. This may affect the relative quantities of the different proteins produced during infection due a transcription gradient, which occurs in all single stranded negative sense RNA viruses. The hRSV genome also contains an overlapping open reading frame in the M2 and L genes that

is not present in hMPV. Thirdly the length of the putative open reading frames (ORF) differs between members of the *Pneumovirinae,* as shown in Table 1.2.

Table 1.2 Lengths in amino acid residues of the putative ORFs of hMPV, hRSV and APVC

Virus	Amino acid length								
	N	P	M	F	M2-1	M2-2	SH	G	L
hMPV	394	294	254	539	187	71	183	236	2005
hRSV	391	241	256	574	194	90	64	298	2165
APVC	394	294	254	537	184	71	175	585	2005

1.7. Virus proteins

1.7.1. The Ribonucleoprotein (RNP) Complex

The 3 major proteins of the RNP complex N, P and L are the minimum trans-acting requirements for *Pneumovirinae* (proteins required for efficient first round replication in the reverse genetics system), and co-localise with the RNA genome in cytoplasmic inclusions of infected cells to form the RNP complex (Grosfeld et al., 1995, Teng & Collins, 1998, Yu et al., 1995).

1.7.2. The N Protein

The N proteins of the *Paramyxoviridae* form an integral part of the RNP complex and is an essential for transcription and replication (Grosfeld et al., 1995, Teng & Collins, 1998, Yu et al., 1995). It binds to viral genomic and anti-genomic RNAs to form an RNAse resistant nucleocapsid and is thought to be responsible for giving the genome its helical structure (Bhella et al., 2002, Easton et al., 2004, Fooks et al., 1993, Meric et al., 1994). The N protein of hMPV contains 394 amino acids with an estimated molecular mass of 43.5KDa, consistent with that of other *Pneumovirinae* (Barr et al., 1991, van den Hoogen et al., 2002). It is highly conserved showing 98-100% amino acid homology between hMPV strains within the same lineage, and 94-96% between lineages and shares 69-89% amino acid identity with different members of the APV, and 42-44% with hRSV (Bastien et al., 2003).

1.7.3. The P protien

The P proteins of the *Paramyxoviridae* also form part of the RNP complex and is also essential for transcription and replication (Grosfeld et al., 1995, Teng & Collins, 1998, Yu et al., 1995). The P protein of hMPV is the major phosphorylated species and is comprised of 294 amino acids, with a predicted molecular mass of 32.5KDa (van den Hoogen et al., 2002). The P proteins of the *Pneumovirinae* exist as homo-dimers and tetramers, and the oligomerisation domain for hRSV P has been mapped to a short amino acid stretch in the central part of the protein (Castagne et al., 2004). Phosphorylation of P is thought to be important for its various functions; although exactly how and why remains to be elucidated. Cellular kinase 2 has been shown to mediate the phosphorylation of specific serine residues in the hRSV P protein, however phosphorylation patterns differ when using this enzyme in vitro indicating other kinases or factors may be involved (Villanueva et al., 1994).

Within each lineage of hMPV, P displays 94.6-100% amino acid homology and 51.6-85.7% between lineages with a majority of amino acid changes occurring at the amino terminal of the protein (Bastien et al., 2003). There is a significant level of sequence divergence between the hMPV P protein and those of other members of the *Pneumovirinae*. The most closely related is that of APV C, which shares 68% amino acid homology. The central region of hMPV P (171-226aa) however represents a highly conserved domain present in the P proteins of all pneumoviruses, and as such a greater level of amino acid identity exists; 80-88% and 63-68% with avian and mammalian pneumoviruses respectively (Bastien et al., 2003). HMPV P also contains a large number of charged amino acids at the carboxyl terminal, another common feature of pneumoviruses (van den Hoogen et al., 2002).

When expressed alone in insect cells or *E.coli* the N protein of hRSV and other *Paramyxoviridae* form nucleocapsid like structures around non-specific cellular RNAs (Meric et al., 1994). When co-expressed with hRSV P however, soluble N-P complexes form which can be co-immunoprecipitated, and non-specific encapsidation is inhibited. It has been suggested therefore that N-P complexes form prior to nucleocapsid assembly, where P acts as a chaperone for N, preventing non-specific binding to cellular RNAs (Castagne et al., 2004, Curran et al., 1995). The carboxyl terminal of both hRSV N and P has been shown to be vital for their interaction; however other regions may be involved. The role of P phosphorylation in N-P interactions still requires clarification. The formation

of hRSV N-P complexes in E.coli is very efficient despite the lack of phosphorylation (Castagne et al., 2004). However, a 40% reduction in the formation of N-P complexes is observed using a reverse genetics system in which the major phosphorylation sites of hRSV P were mutated (Lu et al., 2002). However, structural differences at these mutated sites may account for the reduction in N-P interactions as oppose to a lack of phosphorylation.

1.7.4. The Large (L) Polymerase Protein

The L protein is the major component of the viral RNA dependent RNA polymerase complex, and as such is essential for transcription and replication (Grosfeld et al., 1995, Teng & Collins, 1998, Yu et al., 1995), and thought also to be responsible for mRNA methylation and capping (Colonno & Stone, 1975, Ogino et al., 2005). L is the largest of the hMPV proteins containing 2005 amino acids, and shares 64% amino acid identity with APV-A and 44% with hRSV (van den Hoogen et al., 2002). There are 6 conserved domains within the L proteins of the *Monoegaviriales*, containing 4 core motifs that are highly conserved in all viruses of this order including hMPV (van den Hoogen et al., 2002).

1.7.5. The Transcription Elongation Factor (M2-1)

The first open reading frame of the M2 gene encodes the M2-1 protein, which associates with the RNP complex. The M2-1 gene of hMPV encodes a 187 amino acid protein that shares 84% homology with that of APV-C (van den Hoogen et al., 2002). All the M2-1 proteins of *Pneumovirinae* including hMPV contain a conserved Cys/His zinc finger- like motif that is thought to be essential for its function. M2-1 is an RNA binding protein that interacts with the N and P proteins of the RNP complex. The function of M2-1 has been determined for hRSV through the use of mini genome systems, where it acts as a transcription elongation factor necessary for the full processivity of the viral polymerase (L) and enhances the read-through of intergenic regions, promoting the expression of genes distal to the 3' end of the genome (Collins et al., 1996). Unlike the M2-1 protein of hRSV however M2-1 is not essential for the growth of hMPV in vitro, but was required for detectable levels of growth in vivo (Buchholz et al., 2005).

1.7.6. The RNA Synthesis Regulatory Factor (M2-2)

The second open reading frame of the hMPV M2 gene encodes the putative RNA synthesis regulatory factor (M2-2). The function of M2-2 has only been reported for hRSV, in which

mutants lacking this protein replicated more slowly in cell culture and were highly attenuated in vivo. Mutants resulted in a reduced production of full length genomic RNA, whilst the production of mRNAs was increased. This indicates that M2-2 acts as a regulatory factor switching the polymerase complex from transcriptional to replicational mode (Bermingham & Collins, 1999). HMPV mutants lacking M2-2 were also highly attenuated in vivo, but were capable of replicating in vitro (Biacchesi et al., 2005, Buchholz et al., 2005).

1.7.7. The Matrix Protein (M)

The M protein is associated with the cytoplasmic face of the plasma membrane of infected cells. M has been shown to interact with the RNP complex to inhibit transcription of the viral genome during the late stages of infection, and facilitates assembly of the virion by bringing together the RNP complex and viral envelope proteins. It has been reported that the M protein of hRSV can be found in the nucleus of the infected cells during the early stages of infection where it may inhibit host cell transcription (Ghildyal et al., 2003). During the later stages of infection RSV M is present mainly in the cytoplasm of the cell where it associates with plasma membrane lipid rafts containing the viral surface glycoproteins, this acts as a platform for virion assembly and budding (Ghildyal et al., 2003, Ghildyal et al., 2005, Ghildyal et al., 2002) . The M protein of hMPV contains 254 amino acids and shows a high level of amino acid identity with that of APV (76-87%), but only 37-38% with hRSV. All pneumoviruses contain a conserved hexapeptide at position 14-19 that is also found in hMPV (van den Hoogen et al., 2002).

1.7.8. The Fusion (F) Protein

The F protein of hRSV has been shown to mediate viral penetration in to the host cell following attachment by fusing the virion envelope and the host cell plasma membrane(Walsh & Hruska, 1983). At the later stages of hRSV infection F is expressed on the surface of the infected cell where it can fuse the membranes of neighbouring cells to form syncytia, a characteristic that gives hRSV its name.

hRSV F is synthesised as a full-length inactive precursor, Fo, which trimerises in the rough endoplasmic reticulum (ER). Once in the trans-*golgi* network each protein is cleaved at 2 furin sites into its active form, which is comprised of 2 hetero subunits; F1 and F2, which remain linked via disulphide bonds. This cleavage results in liberating the hydrophobic fusion peptide (Zhao et al., 2000). Although hRSV F is sufficient for the formation of

syncytial, the co-expression of G and SH has been shown to increase its efficiency (Heminway et al., 1994).

The F protein of hMPV contains 539 amino acids with a predicted molecular mass of 58.4Kda (van den Hoogen et al., 2002). The F protein of hMPV is highly conserved and displays 98-100% amino acid homology within subgroups, and 94-95.5% homology between subgroups. The F protein of hMPV shares 81-82% amino acid identity with that of APVC, but only 30-43% identity with hRSV (Bastien et al., 2003). The F protein of hMPV contains the two heptad repeats which have been shown to be necessary for viral fusion in other paramyxoviruses (Baker et al., 1999, Ghosh & Shai, 1999), and 3 potential N-linked glycosylation sites, which are conserved also in APV F proteins(van den Hoogen et al., 2002).

Being a surface protein F is one of the major antigenic determinants of pneumovirinae, and the high level of amino acid homology of the F protein of a particular virus means antibodies generated against F are generally cross-protective for the different strains of the virus in experimental animals (Skiadopoulos et al., 2004). Detection of F antibody has been used in serological assays(Ishiguro et al., 2005, Leung et al., 2005, Skiadopoulos et al., 2004). The ability of hRSV and hMPV to cause repeated infections therefore is thought to lie partly with the other major surface antigen the G protein.

1.7.9. The Small Hydrophobic (SH) Protein

The SH proteins of Pneumoviruses are small integral membrane proteins for which the function is unknown. It is a type II membrane protein that is anchored at the amino terminal, with the carboxyl terminal orientated extracellularly. Several forms of SH are detected in hRSV infected cells, SHo, a full length non glycosylated species; SHg, a full length species containing a single N linked carbohydrate side chain; SHp, a full length species containing an N-linked side chain modified by polylactosaminoglycan, and SHt a non glycosylated species derived from the second methionine in the same ORF (Collins et al., 2001, Olmsted & Collins, 1989). All forms oligomerise to form pentomers. Whether different forms of SH exist during hMPV infection is yet to be determined. The deletion of SH in both hMPV and hRSV reverse genetics systems has shown that the protein is dispensable in vitro, and in some cell lines provides a growth advantage for hRSV, but was attenuated in the upper but not lower respiratory tract of mice and primates (Biacchesi et al., 2005, Biacchesi et al., 2004, Bukreyev et al., 1997, Techaarpornkul et al., 2001).

1.7.10. The Attachment Glycoprotein (G)

The designation of the 7th gene of the hMPV genome as the attachment glycoprotein gene was due to similarities in the properties of the predicted protein, and that of hRSV G (van den Hoogen et al., 2001), identified as the attachment protein, since antibodies specific to hRSV G blocked cell binding (Levine et al., 1987). However, it has been demonstrated using reverse genetics that the G protein of hMPV is not essential for growth in vitro or vivo using a hamster model (Biacchesi et al., 2004) calling in to question its role in receptor binding. Furthermore, in contrast to hRSV G, hMPV G elicits only low levels of neutralizing antibody (Skiadopoulos et al., 2006).

The G protein of hMPV is 236 amino acids in length. This is considerably shorter than that of hRSV A and B, which are 298 and 299 amino acids respectively and for which there is only 15.6% and 15.2% amino acid homology with hMPV G respectively. The G protein of APV C is almost twice its length at 435 amino acids and shares only 19% amino acid homology with hMPV G (Bastien et al., 2004, van den Hoogen et al., 2002)

As with hRSV and APV the G protein of hMPV shows a high degree of nucleotide and amino acid variation both within and between lineages (61% and 33% respectively)(Bastien et al., 2004). This high degree of amino acid variation may contribute to the viruses' ability to evade host immune responses and cause repeated infections throughout life. The extent of variation in the G protein of strains circulating in the UK and world wide is investigated in chapter 4 of this thesis where a more detailed discussion can be found.

G is a type II transmembrane protein containing a hydrophobic region at the N-terminus which serves as both a signal peptide and transmembrane anchor. The remaining two thirds of the protein is orientated externally. Within this external region of the hRSV and APV G proteins there is a conserved region containing a number of motifs, to which neutralising antibodies are directed and is therefore thought to contain the receptor binding site. Four conserved cystine residues are also present in hRSV; 2 either side of this conserved region. Disulphide bonds between these cysteine residues create a cystine loop in which the conserved region is present (Melero et al., 1997). The hRSV G proteins has been shown to form homo oligomers of up to 3 G molecules (Collins & Mottet, 1992, Melero et al., 1997). HRSV mutants lacking this cysteine loop region, however, showed little difference

to wild type virus in terms of the kinetics or efficiency of virus replication in vitro, or in the upper respiratory tract of mice, however, a 3-10 fold reduction of the mutant virus was observed in the lower respiratory tract (Teng & Collins, 2002).

In contrast hMPV G contains only 1 cysteine residue and as yet no conserved region has been identified in the external region, suggesting potential differences in the structure of the protein and its potential binding site.

The G proteins of all *Pneumovirinae* contain a large number of potential O- linked glycosylation sites at serine and threonine residues. For hRSV up to 60% of the molecular weight of the mature form of G has been shown to be due to the presence of carbohydrate (Wertz et al., 1989). Glycosylation has been shown to play a role in immune evasion of hRSV, by masking epitopes and contributing to the antigenic variance of the protein (Melero et al., 1997). The hMPV G ORF also contains up to 5 potential N-linked glycosylation sites. The predicted MW of the polypeptide backbone of hMPV G protein is 26KDa (van den Hoogen et al., 2002). For hRSV G this is approximately 32KDa. The addition of N-linked sugars has shown to increase this to 45KDa, and O-linked sugars to approximately 80 to 90KDa (Wertz et al., 1985, Wertz et al., 1989).

The amino acid sequence also reveals a high proline content of 8.5%. The unusually high potential for O-linked glycosylation and the high abundance of proline residues in the G proteins of pneumovirinae has also been observed in mucins, host glycoproteins present in mucus of the respiratory, gastrointestinal and reproductive tracts (van den Hoogen et al., 2002, Wertz et al., 1985).

The G protein of hRSV is also synthesised in a secreted form due to initiation of translation at the second methionine codon in the same ORF and lacks the entire transmembrane and signal peptide region (Hendricks et al., 1987). The production of a secreted G in hMPV is yet to be shown, however a second AUG is present in the same ORF located immediately downstream of the transmembrane region. The secreted G protein of hRSV is thought to play an important role in immune evasion by mopping up neutralizing antibody and reducing inflammatory responses (Arnold et al., 2004, Hendricks et al., 1987). It has also been demonstrated that soluble hRSV G retains its ability of cell receptor binding, the addition of which to hRSV infected cells inhibits virus spread (Escribano-Romero et al., 2004).

1.8. Virus replication cycle

The replication of Paramyxoviridae takes place in the cytoplasm of the host cell. An over view of the life cycle is shown in Figure 1.4.

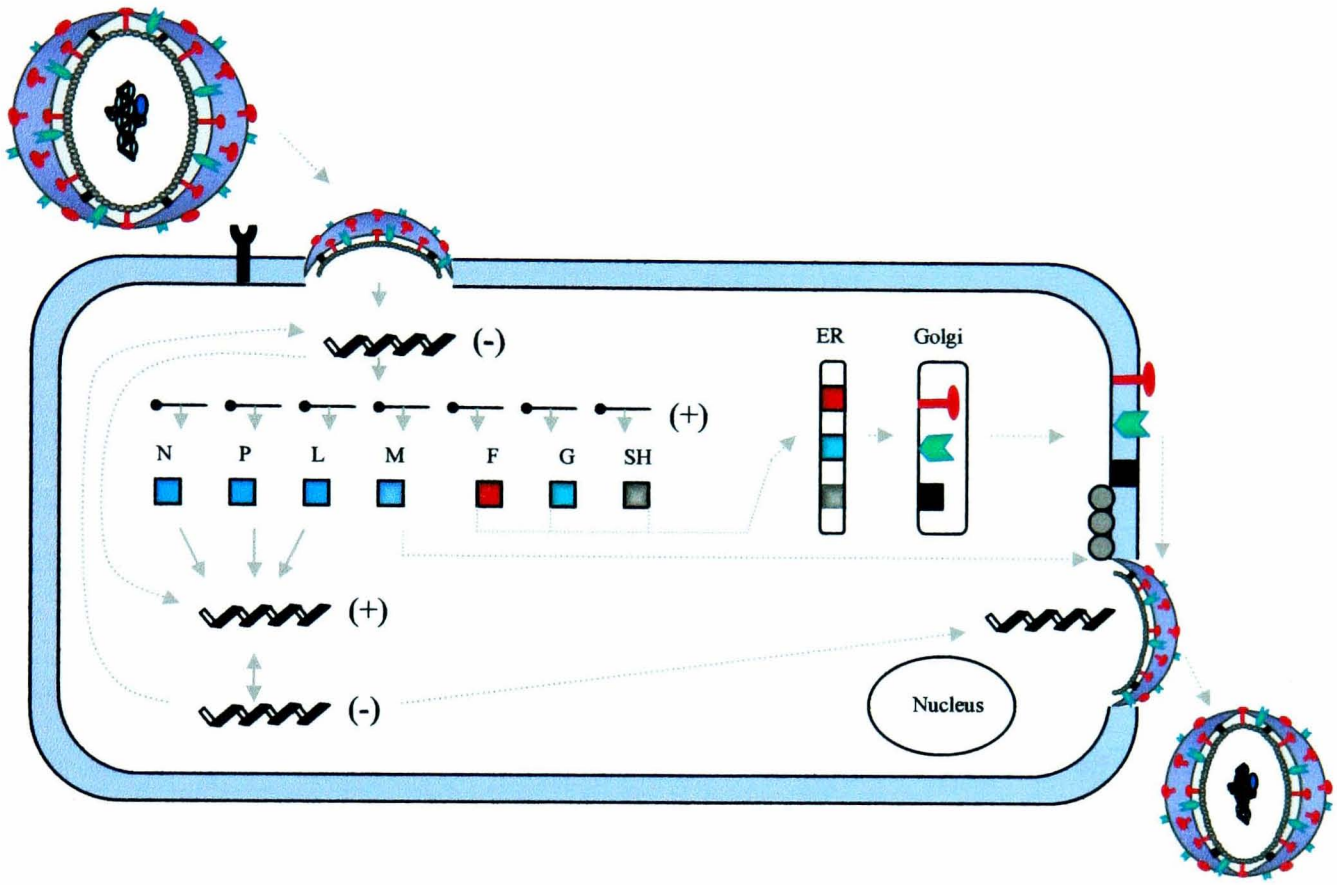


Figure 1.4 Schematic representation of the pneumovirinae life cycle. Adapted from (Lamb & Kolakofsky, 2001).N: Nucleocapsid; P:Phospoprotein; L:Large polymerase; M: Matrix; F: Fusion; G: attachment glycoprotein; SH: small hydrophobic protein. ER: endoplasmic reticulum; Golgi: Golgi apparatus.

1.8.1. Virus attachment and penetration

Attachment of pneumoviruses to the host cell is thought to be mediated via the G protein. As described above however through the use of reverse genetics it has been demonstrated that the G proteins of RSV and hMPV are required for growth in vivo, but are dispensable for growth in vitro indicating alterative mechanisms for attachment may be available by the F protein in vitro (Biacchesi et al., 2005, Biacchesi et al., 2004, Karger et al., 2001, Techaarpornkul et al., 2001, Teng & Collins, 1998). The host cell receptor for hMPV and hRSV is yet to be determined; however hRSV has been shown to bind to certain cellular glycosaminoglycans (GAGs) such as those containing heparin sulphate or condroitin sulphate B (Feldman et al., 2000, Feldman et al., 1999, Hallak et al., 2000a, Hallak et al., 2000b, Krusat & Streckert, 1997, Martinez & Melero, 2000, Shields et al., 2003).

The pneumovirinae enter the host cell by fusion of the host cell plasma membrane with the envelope of the virion. The fusion event is mediated by the Fusion (F) protein at neutral pH. Whilst fusion by hRSV can be mediated by F alone, there is evidence indicating that SH and G may enhance the fusion process (Heminway et al., 1994). After membrane fusion the nucleocapsid dissociates from the matrix protein by an unknown mechanism and is released into the cytoplasm of the cell, where transcription and replication of the viral genome occurs.

1.8.2. *Pneumovirinae* Transcription

The current model of transcription and replication for *Pneumovirinae* is consistent with that for all non-segmented negative sense viruses and occurs entirely in the cytoplasm of the infected cell.

The polymerase complex (N, P and L) attaches to the genome at the leader sequence at the 3' end of the genome, and initiates transcription at the gene start (GS) signals located at the beginning of each transcribable gene. The polymerase complex continues along the genome synthesising the mRNA strand until the gene end (GE) signal is reached at which point transcription is terminated. Following the GE signal *Pneumovirinae* genomes contain a run of U residues, which marks the position at which the mRNA is polyadenylated and released from the genome-polymerase complex. During the transcription process the nascent mRNA is also methylated, allowing the gene to be translated by host cell ribosomes, located either on the endoplasmic reticulum, or by free ribosomes in the cytoplasm, depending on the nature of the protein product (Collins et al., 2001, Easton et al., 2004).

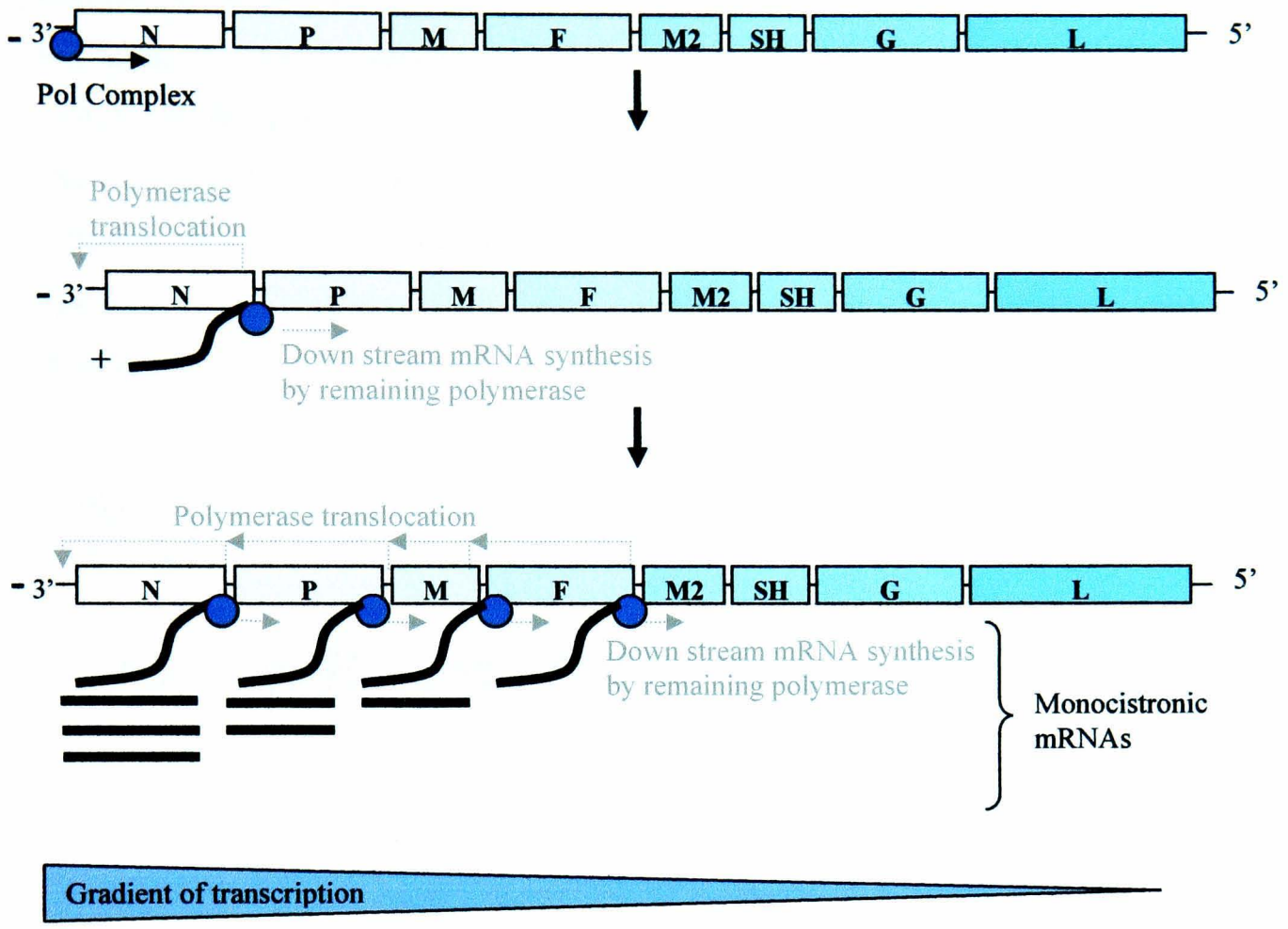


Figure 1.5: Schematic representation of the *Pneumovirinae* transcription process.

At the GE sequence approximately only 50% of the active polymerase molecules are able to remain attached and move along the genome without transcribing until they reaches the next GS signal upon which transcription reinitiates, at the next GE signal the same process occurs where approximately 50% of the polymerase will remain attached and go on to transcribe the next gene, the remaining 50% will dissociate from the genome. The dissociated polymerase translocates back to the leader sequence at the 3' end where it can reattach and initiate transcription of the first gene. The polymerase is unable to reattach at any other location other than the 3'leader sequence. This means that the genes proximal to the 3' leader sequence are transcribed more frequently than those, which are located at the 5' end resulting in a transcription gradient (Easton et al., 2004, Krempl et al., 2002). The association of the M2-1 protein with the polymerase complex during transcription inhibits dissociation of the polymerase from the genome, therefore allowing genes distal to the 3' end to be transcribed (Buchholz et al., 2005).

1.8.3. Genome Replication

During replication the polymerase complex binds to the leader sequence at the 3' end of the genome, where synthesis of the anti-genome initiates (Fearns et al., 2002). Once committed the polymerase continues to the end of the genome. The polymerase then uses the anti-genome as a template to synthesis genome. In order to do this the polymerase complex binds to the anti-genome 3' end, where the sequence complementary to the genome trailer sequence acts as a binding site for the polymerase, and promotes replication.

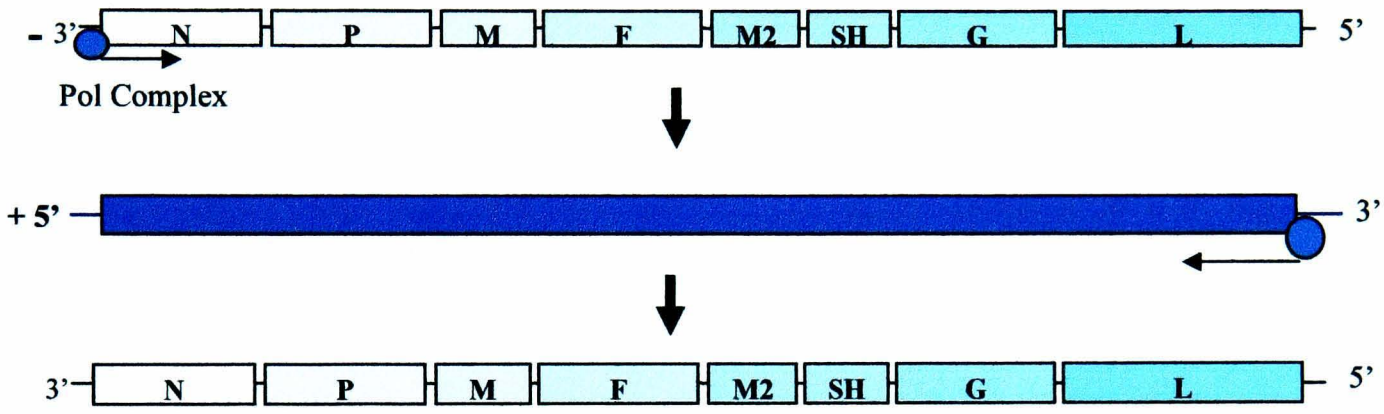


Figure 1.6: Schematic representation of *Pneumovirinae* genome replication

1.8.4. Transcription versus Replication

It is not yet clear how the polymerase switches between transcription and replication. The M2-2 protein has been shown to play a role, although how M2-2 functions is not known (Bermingham & Collins, 1999). In accordance with other members of the *Monoegaviriales,* replication may also be dependent of RNA encapsidation, which requires ongoing protein synthesis. Therefore increasing levels of N protein expression may influence this switch, although there is conflicting data suggesting that the increase in N stimulates transcription rather than replication (Fearns et al., 1997, Lamb & Kolakofsky, 2001).

1.8.5. Virion Assembly

The assembly of the nucleocapsid takes place in the cytoplasm where the components of the polymerase complex N, P and L associate with genomic RNA to form the helical RNP structure. Both genomic and antigenomic RNA is encapsidated, however mRNAs are not. It is therefore assumed that the 5' trailer sequence of genomic RNA and 5' leader of

antigenomic RNA contains a specific sequence for encapsidation (Easton et al., 2004, Mink et al., 1991).

The second stage of virus assembly; the envelope takes place at the cell surface. The viral membrane proteins are synthesised in the ER. The folding of which involves numerous cellular enzymes. Once folded the proteins are transported out of the ER through the Golgi apparatus, where further maturation in the form of glycosylation or protein cleavage occurs. Mature forms of the proteins are transported to the plasma membrane where they exist in lipid rafts(Brown et al., 2004, Collins & Mottet, 1992, Olmsted et al., 1989, Rixon et al., 2004).

The third stage of virus assembly involves the association of the RNP with the surface glycoproteins which is thought to be mediated by the M protein. M is thought to interact with the cytoplasmic tails of the surface proteins during their passage through the Golgi apparatus(Ghildyal et al., 2005, Ghildyal et al., 2002, Marty et al., 2004). M has also been shown to associate with the RNP complex in cytoplasmic inclusions present in infected cells near to the plasma membrane (Ghildyal et al., 2002). Aggregation of M proteins bound to the RNP and glycoproteins brings the two components together resulting in progeny virions which bud from the surface of the cell. In polarised cells such as those lining the respiratory tract virus is released from the apical side into the airways (Roberts et al., 1995).

1.9. Clinical Manifestations

HMPV has been associated with acute respiratory tract infections (ARTI) in people of all ages, with more severe disease occurring in young children, the elderly and immunocompromised individuals (Boivin et al., 2002, Boivin et al., 2003, Falsey et al., 2003, Pelletier et al., 2002).

Clinical features of hMPV are virtually indistinguishable from those of hRSV, although the course of illness is usually less severe(Boivin et al., 2003). Symptoms include high fever, severe cough, difficulty breathing, and wheezing. The most commonly reported diagnosis associated with hMPV is bronchiolitis with or without pneumonitis (Boivin et al., 2003). HMPV has also been associated with COPD and asthma exacerbations(Martinello et al., 2006, Rohde et al., 2005, Williams et al., 2005a). One study showed that as many as 14%

of hMPV cases triggered asthma exacerbations following infection in children with a medical history of asthma(Williams et al., 2004). HMPV has also been detected in patients with otitis media (Schildgen & Simon, 2005, Suzuki et al., 2005, Williams et al., 2006).

Hospitalization rates due to severe hMPV infections peak in children of 3-6months of age(Boivin et al., 2003, van den Hoogen et al., 2003). As with hRSV, children with underlying medical conditions are more prone to severe illness due to hMPV infection leading to increased rates of hospitalization in this group. One study in north America suggest that 25-33% of hMPV cases requiring hospitalisation occurred in children with underlying conditions, which ranged from cardiopulmonary problems to immunosuppression (Boivin et al., 2002, Esper et al., 2003). However the risk factors for severe hMPV infection have not been fully investigated.

Co-infection of hMPV and hRSV were associated with increased severity of disease compared with hRSV alone. In this study 70% of infants hospitalised with hRSV bronchiolitis were also infected with hMPV, and 90% of those requiring mechanical incubation were co-infected (Greensill et al., 2003, Semple et al., 2005). During the recent SARS Coronavirus outbreak in 2003 hMPV was identified as the most common co-pathogen and a possible synergistic effect on disease severity has been postulated, however synergistic effects were not found in Macaques co-infected with SARS CoV and hMPV, but this remains to be investigated further (Fouchier et al., 2003, Kuiken et al., 2003b, Kuiken et al., 2004).

HMPV infections in adults have been less well studied than those in children. However, a few studies have associated hMPV with influenza like illness and colds in healthy adults. HMPV was identified in 2% of adults in England with Influenza like illness (ILI) where no other viral pathogen was detected (Stockton et al., 2002), and in 4.1% of adult patients who attended the emergency room with community acquired pneumonia and chronic obstructive pulmonary disease (COPD) exacerbations over 2 winter seasons in Canada (Hamelin et al., 2005a). In a retrospective study of 10 patients over 65 years of age hospitalised with an hMPV infection, 4 were diagnosed with pneumonitus and 2 died (Boivin et al., 2002).

HMPV has also been associated with the death of a child who suffered with a severe lower respiratory tract infection following a hematopoietic stem cell transplant(Cane et al., 2003),

and an immunocompromised child who was repeatedly co-infected with both subtypes of hMPV (Pelletier et al., 2002). A case of fatal encephalitis has also been associated with hMPV infection following the detection of hMPV RNA in the brain and lung tissue following post mortem (Schildgen et al., 2005).

1.10. Immune Response to pneumovirinae

Protection against and recovery from pneumovirus infection is mediated largely by the host immune response. It has been shown that children with primary or acquired immunodeficiency diseases fail to clear hRSV infection and virus may be shed for several months, compared to immunocompetent children where clearance is achieved within 3 weeks (Chandwani et al., 1990) it is likely that this is similar for hMPV, the only study thus far to comment of viral shedding in children showed that virus could be detected by RT-PCR for up to 3 weeks in otherwise healthy children (Ebihara et al., 2004). In immunocompromised adults, such as those having undergone bone marrow transplantation, serious hMPV and hRSV infections have also been reported, with many leading to death (Englund et al., 2006, Englund et al., 1988, Fouillard et al., 1992, Harrington et al., 1992). In studies of hRSV infection in mice, CTL and antibody responses both play a role in the eradication of virus from the respiratory tract.

1.10.1. Antibody responses

Post infection sera contains antibodies against the various structural proteins of the virion, which provides many opportunities for the development of sero-diagnostic assays, however typically only antibodies targeted towards the F or G proteins of pneumovirinae have been shown to neutralize virus in vitro. Recent work by Skiadopoulos et al (2006) however indicated that in contrast to other members of this subfamily only low levels of neutralizing antibody is generated against the hMPV G protein, and therefore the hMPV F protein appears to be the sole antigen against which protective antibodies are generated (Skiadopoulos et al., 2006).

Neutralizing antibodies targeted towards the F proteins of *Pneumovirinae* appear to target conformational epitopes, and have been mapped primarily to the F1 subunit of the protein (Lopez et al., 1993, West et al., 1994). Antibodies generated against the F protein of hMPV have been shown to be reactive against both lineages (Skiadopoulos et al., 2004). In contrast antibody recognition of hRSV G appears not to be dependent of conformational

epitopes (Martinez et al., 1997), are often not reactive against viruses from both lineages, and are normally classified as group specific, which are more likely to recognise non-glycosylated species of G, or strain specific, which are often dependent of the presence of glycosylation (Martinez et al., 1997). It is not yet clear whether antibodies against hMPV G behave in a similar manner.

New born infants possess maternally derived antibody against hRSV which offers protection against severe illness in a majority of individuals. These maternally derived antibodies however only offer a limited amount of protection and infection with hRSV still occurs (Glezen et al., 1981, Ogilvie et al., 1981). Antibodies are produced in high titre in older infants and young children where maternal antibody has declined. However in infants 1-8 months of age only a limited antibody response is mounted due to immunological immaturity and suppressive effects of maternal antibody (Murphy et al., 1986, Murphy et al., 1988).

Secreted IgA plays an important role in protecting against reinfection. However this does not last long following primary infection and often fails to neutralize virus (in vitro)(McIntosh et al., 1978). Repeat infections induce higher levels of IgA which can be sustained for longer periods; however this remains only partially protective as adults continue to become symptomatically infected (Hall et al., 1991, Walsh & Falsey, 2004). Serum antibody persists at much higher titres for longer; inefficient transuduction of IgG into the respiratory tract however means it offers little protection.

1.10.2. Cellular immune response

Clearance of human *Pneumovirinae* infection has been associated with an increase in CTL response. Nude mice infected with hRSV have been shown to persistently shed virus, which is cleared upon adoptive transfer of primed T cells (Cannon et al., 1987).

The N, M, SH, and F proteins of human *Pneumovirinae* have been shown to stimulates specific T cell responses(Cherrie et al., 1992, Nicholas et al., 1990). In contrast a CTL response against the G protein has not been detected (Bangham et al., 1986, Cherrie et al., 1992) and this is reflected in the pattern of the T helper cell response which is biased towards Th-2, while that of F is biased towards a Th1 response(Hancock et al., 1996) .Overall pneumovirus infection is characterised as a Th-1 response.

Infection of epithelial cells and macrophages of the respiratory tract with human *Pneumovirinae* induces transcriptional activation of cellular genes involved in inflammatory and immune responses. Increases in pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, and RANTES result in the recruitment and activation of macrophage, neutrophils, eosinophils CTLs and NK cells involved in viral clearance either by direct attack of infected cells or release of degradative enzymes.

Abberant immune responses to hRSV are thought to be involved in the pathogenesis of hRSV infection in young babies, where narrow airways are easily obstructed as a result of inflammation and oedema (McIntosh & Fishaut, 1980, Murphy et al., 1990) . Adverse immune responses have also been observed during vaccine development in which children vaccinated with a formalin inactivated hRSV suffered significantly more severe disease than unvaccinated children upon natural infection (Chin et al., 1969, Kapikian et al., 1969, Prince et al., 1986). It is unknown whether immunopathogenesis is involved in the severity of hMPV infections.

1.11. Pathogenesis

A number of small animals (hamsters, cotton rats, mice and ferrets) and primates (chimpanzees, cynomologous and rhesus macaques, and African green monkeys) have been evaluated for their susceptibility to hMPV and their ability to support viral replication in order to develop a model for the investigating of hMPV pathogenesis, antiviral compounds and vaccines (Alvarez & Tripp, 2005, Darniot et al., 2005, Hamelin et al., 2005b, Kuiken et al., 2004, van den Hoogen et al., 2001, Williams et al., 2005b). Virus replication has been reported in the upper and lower respiratory tracts of many of these, however, only BALB/c mice, cynomologous macaques and chimpanzees have been reported to display clinical symptoms (Alvarez et al., 2004, Hamelin et al., 2005b, Kuiken et al., 2004, Skiadopoulos et al., 2004, van den Hoogen et al., 2001) .

Studies conducted by Alvarez et al (2004) show hMPV replicates in a biphasic growth pattern in BALB/c mice with peak titres occurring at days 7 and 14. However, only monophasic replication has been reported by other groups with peak lung titres occurring on day 4 or 5 (Hamelin et al., 2005b).

Alverez et al (2004) also reported that infectious virus could be recovered from the lungs of infected mice up to 60 days post infection, and genomic RNA could be detected by RT-PCR up to 180 days pi. Other groups however have not reported the same length of viral persistence, (Hamelin et al., 2005b) reported similar levels of virus replication, but could only recover / detect virus up to 3 weeks pi.

The persistence of hMPV observed by Alvarez et al (2004) occurred despite the presence of neutralizing antibodies, which protected the mice from subsequent challenge. This same group went on to demonstrate that this persistence was associated with aberrant immunity, and impaired viral clearance, due to the delayed onset of CTL responses compared to that of hRSV in this same mouse model. Furthermore the depletion of T cells or NK cells resulted in increased levels of virus replication in the lungs (Alvarez & Tripp, 2005).

Cotton rats, ferrets and Syrian golden hamsters have also been shown to support high titre replication of hMPV with peak virus lung titre occurring on days 4/5 pi and complete viral clearance within 2 weeks. The induction of neutralizing antibodies was also reported, which were protective against subsequent challenge in ferrets and hamsters but only partially protective in cotton rats (Hamelin et al., 2005b, MacPhail et al., 2004).

In cynomologous macaques and African green monkeys peak lung titres were observed between days 4 to 7 pi with shedding lasting up to 11 days pi. Clinical symptoms were not noted in African green monkeys, however cynomologous macaques were reported to have had slight loss of appetite and rhinorrhea on the days of peak virus titre (Kuiken et al., 2004, MacPhail et al., 2004).

Using immunohistological techniques hMPV was detected primarily around the apical plasma membranes of ciliated epithelial cells in multi focal groups (adjacent cells). hMPV was not detected in goblet or basal cells of the respiratory tract nor in other tissues investigated (brain, heart, lung, liver) of infected animals (Hamelin et al., 2005b, Kuiken et al., 2003b, MacPhail et al., 2004).

Similar pathological changes were observed in the respiratory tracts of monkeys, cotton rats and BALB/c mice. Typically, the loss of cilia in the epithelium was noted to occur in multifocal lesions throughout the length of the respiratory tract from the larynx to the bronchiols, and some surrounding alveoli. Pulmonary inflammation was observed in these

animals with the translocation of neutrophils in the epithelium and sub-mucosa, with increased mucus production noted (Kuiken et al., 2004, MacPhail et al., 2004, Wyde et al., 2005). In BALB/c and cotton rats inflammation was associated with increases in cytokines such as IL-2, IL-8, IL-4 and IFNγ. In BALB/c mice inflammatory changes were still present in the lungs more than 21 days pi (Wyde et al., 2005).

A number of studies have shown that for hRSV severe disease is associated with high levels of inflammatory cytokines and chemokines. Due to the similarity of the symptoms observed during hMPV and hRSV infections, it was therefore postulated that similar inflammatory responses may be induced during infection. Studies comparing the cytokine responses of these two viruses however have shown that hMPV is a much poorer inducer of inflammatory cytokines, compared to hRSV. In BALB/c mice hMPV induced lower levels of IL-1, IL-6, and TNFα compared to hRSV, but was a more potent inducer of granulocyte macrophage colony stimulating factor, INFα, and INFγ (Darniot et al., 2005). Similar findings were also observed by (Laham et al., 2004), who analysed the cytokine responses of infants infected with hMPV and hRSV. As with the previous studies hMPV elicited significantly lower levels of inflammatory cytokines compared with hRSV (IL-12, TNFα, IL-1, IL-6, and IL-8). Furthermore the cytokine profiles of infants with hMPV requiring hospitalisation were no different to those who did not require hospitalization. This indicates that the symptoms elicited by hMPV are mediated via an independent mechanism to that of hRSV.

1.12. Vaccine Development

Several different strategies for vaccine development against hRSV have been investigated. However, only a few have made it to clinical trials. The search for a hRSV vaccine has been hindered considerably by a study in which formalin inactivated hRSV tested in young children, resulted in enhanced disease upon natural infection. Concerns exist that a similar problem may be experienced with hMPV (Chin et al., 1969, Kapikian et al., 1969, Prince et al., 1986). For hRSV however live attenuated vaccines appear to be the best approach, and no enhanced disease associated with these vaccine candidates has been reported.

Reverse genetics systems provide useful tools for the development of such vaccines, where mutations can be engineered to suit specific needs. It is through the use of reverse genetics systems that a majority of hMPV vaccine development has been focused.

Recombinant hMPV viruses lacking either the G (ΔG) or G and SH (ΔG+SH) genes replicated efficiently in vitro but displayed at least a 600 fold reduction in replication in the lungs of hamsters when administered intranasally. High levels of neutralizing antibodies however were induced, and the hamsters were protected upon challenge with wild type virus (Biacchesi et al., 2004). More recently African green monkeys were also administered recombinant hMPV viruses lacking either G, SH or M2-2. Little effect on virus replication was observed with the ΔSH virus, however both the ΔG and ΔM2-2 viruses were highly attenuated with 3200fold, and 4000fold reductions in virus replication observed in the lower respiratory tract respectively. High levels of neutralizing antibodies were induced and the monkeys were protected upon challenge with wild type virus (Biacchesi et al., 2005). ΔG and ΔM2-2 recombinant hMPV viruses therefore provide promising vaccine candidates.

Chimeric viruses generated through reverse genetics have also provided possible multivalent vaccine candidates. In one study recombinant hPIV1 expressing the F gene from hMPV induced neutralizing antibodies in hamsters against both viruses and protected against both PIV1 and hMPV during challenge with wild type viruses (Skiadopoulos et al., 2004). A similar study using a recombinant bovine PIV3 virus expressing the F proteins of hPIV3 and hMPV also induced neutralizing antibodies and protected against challenge with wild type hMPV and hPIV3 in hamsters and African green monkeys (Tang et al., 2005, Tang et al., 2003). In both these studies it was noted that the hamsters were protected against both subtypes of hMPV indicating the F gene provide a substantial level of cross-reactive neutralizing antibodies.

In a different approach (Herd et al., 2006) used predictive bioinformatics, peptide immunization, and functional T cell assays to identify cytotoxic T cell epitopes that would be recognised by mice. In this study several T cell epitopes were identified in the N, M2-2, SH and G proteins of hMPV. Vaccination of mice with the corresponding peptides reduced both viral titre and lung pathology. Increased T helper cell Type 1 responses were observed in the lungs and pulmonary lymph nodes measured by an increase in IL-12 and IFN-γ. The epitopes identified in the G and SH proteins varied between subtypes, and therefore may be less suitable for vaccine development than the N and M2-2 peptides, which represent conserved epitopes present in all subtypes of hMPV.

1.13. Antiviral Therapy

Currently there are no drugs or compounds available for the treatment of hMPV. A number of antiviral compounds however have been evaluated and are licensed for use against hRSV, and therefore tested for use against hMPV.

Ribavirin, a nucleoside analogue of guanosine has been used for the treatment of hRSV pneumonitis in young children. In vitro studies have shown ribavirin has a similar level of antiviral activity against hMPV and hRSV (Wyde et al., 2003). Moreover hMPV infected BALB/c mice had significantly reduced levels of hMPV virus replication in the lungs, and reduced pulmonary inflammation when treated with ribavirin (Hamelin et al., 2006). In this same study glucocorticoids, which are powerful suppressors of innate immunity, were also evaluated. While no decrease in viral replication was observed in the lungs of mice treated with this drug, lung inflammation and weight loss were significantly reduced. The use of ribavirin, and glucocorticoids used separately or in combination may provide a powerful antiviral treatment against hMPV infection.

NMS03, a sulphated sialyl lipid and heparin, another sulphated compound have also been shown to be effective against hMPV in vitro, especially when administered before or during viral attachment and penetration (Wyde et al., 2004), also suggesting that hMPV may have a similar mode of attachment as hRSV.

Intravenous immunoglobulins (IVIG), used for prophylactic treatment against hRSV, were also shown to inhibit the growth of hMPV in vitro (Wyde et al., 2003), and may therefore be of some benefit in vivo. Indeed Alvaerez et al demonstrated that affinity purified α-hMPV IgG when passively transferred in to naïve BALB/c mice reduced the levels of hMPV replication when challenged with virus. The humanised monoclonal antibodies targeted towards the hRSV F protein (palivizumab) have been shown to decrease the severity of lower respiratory tract infections for both subtypes of hRSV. These antibodies tested in vitro against hMPV had however no antiviral activity (Wyde et al., 2003). Due to the conserved nature of the hMPV F gene, hMPV specific monoclonal antibodies targeted to this protein could be effective.

Recently antiviral activity through RNA interference (RNAi) has been studied. RNAi is a mechanism of sequence specific silencing through the formation of dsRNA molecules, and is well recognised as being instrumental in the antiviral responses of plants and insects. The introduction of virus specific small interfering RNAs (siRNA) into cells has been shown to be an effective therapeutic against mammalian viruses in vitro and vivo for a wide range of viruses (Barik, 2004, Bitko et al., 2005, van Rij & Andino, 2006). In vivo studies targeting hRSV and PIV using an intranasal delivery system in BALB/c mice, showed reduced lung titres and pulmonary pathology (Bitko et al., 2005), and may therefore also be effective against hMPV.

1.14. Detection Methods

One of the reasons that hMPV was not identified before 2001 is that growth of this virus in cell culture is particularly fastidious, requiring cell lines not commonly used in diagnostic laboratories, trypsin, and incubation periods of up to 3 weeks, often without the production of cytopathic effect (CPE). Some studies have reported the successful growth of hMPV in LLC-MK2, tertiary monkey kidney and Vero cells. CPE has been reported to occur more than 10-14 days post infection (pi) and varies from the formation of syncytia to focal rounding and cell destruction.

The detection of viral antigens by immunofluorescence (IF) provides a rapid diagnosis for a wide range of possible pathogens and is used routinely in diagnostic laboratories. For hMPV, and many other pathogens however, IF has proved to be not as sensitive as other techniques such as reverse transcription – polymerase chain reaction (RT-PCR) (Casiano-Colon et al., 2003, Ebihara et al., 2005). A number of block-based and real-time RT-PCR assays have been described which target mainly the L, P, N or F genes of hMPV.

A number of serological assays such as enzyme linked immunoabsorbance assays (ELISA) and IF against hMPV infected cells or hMPV recombinant proteins have been reported, but only provide a means of retrospective diagnosis. However are useful for confirmatory diagnosis and seroprevalence studies.

1.15. Scope of this thesis

As stated previously, at the start of this work very little of this information was known about hMPV in terms of its contribution to burden of disease and epidemiology, and still enormous gaps in our knowledge exist. This thesis aims to address the questions related to the epidemiology and prevalence of hMPV in different populations within the UK.

- What is the burden of illness due to hMPV infection?
- When does it circulate?
- What age group is most at risk of infection?
- What illnesses is it associated with?
- Which strains are circulating in the UK?
- Is strain diversity important, and what drives it?

By addressing these important questions we can ask whether hMPV vaccine production is worth while, and identify what factors should be considered in the pursuit of a suitable vaccine candidate.

Chapter 2

Materials and Methods

2.1. Clinical Specimens Receipt and Analysis

2.1.1. Routine Surveillance Samples

Respiratory clinical samples, mainly combined nose and throat swabs or nasopharyngeal aspirates were taken from patents either in the community presenting with Influenza like Illness (ILI), or hospitalised children presenting with acute respiratory tract infection. Swabs were put in to 2ml of virus transport medium (VTM) [VTM: 10g veal infusion broth, 2g bovine albumin fraction V, 400ml sterile distilled water, 0.8 ml gentamicin sulfate solution (50 mg/ml) and 3.2 ml amphotericin B (250 µg/ml)], and posted to the HPA Colindale for analysis. Upon receipt, swabs were removed and 200µl of 250µg/ml Amphotericin B Fungizone (Invitrogen) was added. 150µl of the sample was aliquoted in to 50µl of L6 extraction buffer (Severn Biotech). Aliquots and the remaining original sample were stored at -80^0C until testing.

Samples were processed, extracted and tested for influenza and respiratory syncytial virus first as part of routine surveillance. The remaining cDNA was stored at -20^0C until tested for hMPV. The samples were tested for hMPV by F gene PCR as described later was carried out in batches of 20, with the appropriate controls.

2.1.2. Samples submitted for Severe acute Respiratory Syndrome (SARS) investigation

Respiratory samples sent to the HPA Colindale for investigation in to the SARS outbreak from returning travellers in 2003 were processed in a category 3 laboratory. 150ul of sample was aliquoted in to 850ul of L6 lysis buffer (Severn Biotech). Aliquots and the remaining original sample were stored at -80^0C until testing. Nucleic acid extraction was carried out using the manual Boom method described previously (Boom et al., 1990, Stockton et al., 1998) . cDNA was generated as described below and stored at -20^0C until tested.

2.1.3. Samples collected from immunocompromised patients

An aliquot of the original patient sample taken and previously tested for other respiratory pathogens at the Royal Free Hospital, were sent on wet ice to Colindale HPA for hMPV screening by RT-PCR. 150µl of the sample was aliquoted in to 50µl of L6 extraction buffer

(Severn Biotech). Aliquots and the remaining original sample were stored at –80^{0}C until testing.

2.1.4. Serum Samples

Serum samples were sent to the HPA Colindale by post and were aliquoted and stored at –20^{0}C until testing.

2.2. Mammalian Cell Culture Techniques

2.2.1. Maintenance

Mammalian cells (Table 2.2) were maintained on Minimum Essential Medium (MEM) (1X) liquid containing Earle's salts, and 25 mM HEPES (Invitrogen) supplemented with a final concentration of 10% Foetal Bovine Serum (Invitrogen), 2mM Glutamax I (Invitrogen), 50μg/ml Gentamicin (Invitrogen), and 1x MEM Non- Essential amino acid solution (Sigma). Cells were trypsonised with Trypsin EDTA (Invitrogen).

2.2.2. Virus Growth from Clinical Samples

Tissue culture tubes or 6 well plates were seeded with LLC-MK2 cells (1x 10^6 Cells/ml for tubes and 1x 10^6 Cells/well for plates) in growth medium (described above), and incubated at 37^0C until cells were 80-100% confluent. Growth medium was removed and the cells washed twice in PBS. 200-500μl of the clinical specimen was inoculated on to the cells and adsorbed at room temperature for 1 hour. The inoculum was removed and replaced with 1ml of post inoculation media (Growth medium without the FBS, supplemented with 1μl/ml of 2.5% Trypsin (Biowhittica)), and incubated at 33^0C. Tubes were placed on a roller. Cells were monitored daily for CPE. On days 2 and 7 post inoculation 1μl of fresh trypsin was added to the medium. Cells were harvested by scraping the cells in to the medium and sonicating for 15 minutes on days 7 or 14 post inoculation or when CPE was evident. The harvested material was passaged on to a fresh monolayer of LLC-MK2 cells (80 – 100% confluent) in a tissue culture tube or 25cm^3 tissue culture flask as described above. Some material was saved and analysed by hMPV PCR 1 described below.

2.2.3. Production of hMPV Infected Cells for Analysis by Immunofluorescence

The hMPV isolates (1x A subtype and 1x B subtype) used in this study to validate antiserum were kindly provide by Dr Alison Bermingham (HPA, Colindale), in the form of infected cell culture supernatant. The original clinical isolates originated from patients with ARTI in Northern Ireland, propagated oh RMK cells at Belfast HPA.

Coverslips were placed in the bottom of each well of a 24 well tissue culture plate, and seeded with LLC-MK2 cells (5x10^5 cells/ well) in growth medium and incubated at 37^0C

until cells were 80-100% confluent. Growth medium was removed and the cells washed twice in PBS. 250µl of the hMPV isolate (unknown quantity of virus) was inoculated on to the cells and adsorbed at room temperature for 1 hour. The inoculum was removed and replaced with 1ml of post inoculation media (Growth medium without the FBS, supplemented with 1µl/ml of 2.5% Trypsin (Biowhittica)), and incubated at 37^0C 5%CO_2. Cells were monitored daily for CPE. On days 2, 7 and 14 post infection 1µl of fresh trypsin was added to the medium.

On days 7, 14 or 21 post infection the medium was removed and the cells washed with 1ml PBS then fixed with 80% ice cold acetone in PBS for 10 minutes at -20^0C. The acetone was removed and the cells air dried at room temperature for 30 minutes.

Cells were washed 3 times for 5 minutes with PBS containing 0.1% (v/v) TWEEN 20, and blocked with 1% BSA (v/v) in PBS for 15 minutes. Cells were washed as before then incubated with 200µl primary antibody (Rabbit α-hMPV N-FL or rabbit α-hMPV Total) diluted 1:25 in PBS containing 0.1% (v/v) TWEEN 20 and 1% BSA (v/v) for 30 minutes at 37^0C. Cells were washed as before and incubated with Secondary FITC conjugated antibody (swine α-rabbit IgG FITC) diluted 1:40 in PBS containing 0.1% (v/v) TWEEN 20 and 1% BSA (v/v), 0.01% Evans Blue counter stain and incubated for 30 minutes at 37^0C.

An FITC conjugated rabbit α-hMPV N-FL IgG was also evaluated for its use as a direct immunofluorescent agent. In this instance fixed cells were incubated with 200µl of the conjugated antibody diluted 1:25 in PBS containing 0.1% (v/v) TWEEN 20, 1% BSA (v/v) and 0.01% Evans blue counter stain for 30 minutes at 37^0C (The conjugate was kindly prepared by Dr Dhan Samuels at the HPA Colindale).

Fluorescence was analysed using a Zeiss Axiovert 200 Inverted Microscope. Images were captured with a Zeiss AxioCam HRc and Axio vision software.

2.2.4. Production of hMPV Infected Cells for Analysis by Western Blot.

6 well tissue culture plates were seeded with LLC-MK2 cells (1x 10^6 Cells/well) in growth medium, and incubated at 37^0C until cells were 80-100% confluent. Growth medium was removed and the cells washed twice in PBS. 500µl of the inoculum (described in 2.2.3) was adsorbed on to the cells at room temperature for 1 hour. The inoculum was removed

and replaced with 1ml of post inoculation media (Growth medium without the FBS, supplemented with 1µl/ml of 2.5% Trypsin (Biowhittica)), and incubated at 37^0C 5%CO_2 and monitored daily for CPE. On days 2 and 7 post inoculation 1µl of fresh trypsin was added to the medium. On day 14 cells were harvested by scraping the cells in to the cell culture medium. The media was transferred to a 2ml microcentrifuge tube and the cells pelleted by centrifugation. The cell pellet was resuspended in 1ml of 1x Nupage LDS sample lysis buffer (Invitrogen) and 1x Nupage Sample reducing agent (Invitrogen) containing DTT and heated for 10 minutes at 100^0C. 20µl of the lysed sample preparation was resolved on a 4-12% Nupage Novex Bis-Tris acrylamide gels(1.5mm thick) (Invitrogen), for approximately 1 hour at 180V with 1 x MOPS or 1 x MES Buffer (Invitrogen) using the XCell SureLock Mini Cell electrophoresis equipment (Invitrogen). Transfer onto nitrocellulose and detection was performed as described in 2.6.2.

2.3. Molecular Techniques

2.3.1. PCR anti Contamination Precautions

To prevent any carryover contamination, nucleic acid extraction and reverse transcription, PCR reaction mix set up, thermocycling, and gel analysis were all conducted in separate rooms each with its own dedicated equipment and clothing. All pippetting was carried out with filtered tips. Addition of primary products to a secondary round PCR mix was carried out in UV equipped PCR workstations (LabCaire; Scientific Laboratory Supplies). When large runs of samples were tested negative water controls were placed every 6 samples so carry over could be detected. Positive controls were at the end of every run.

2.3.2. Nucleic Acid Extraction

Total nucleic acid was extracted from 150µl volume of sample in 50µl of L6 Nucleic Acid Extraction Buffer (Severn Biotech Ltd) using the MagNA Pure LC automated system (Roche Diagnostics) in conjunction with the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics) according to the manufacturers instructions. Total nucleic acid was eluted in a 100µl volume of elution buffer.

2.3.3. cDNA Synthesis

For reverse transcription 22.2µl of the total nucleic acid was added to 17.8µl reaction mixture to create a final concentration of 20mM Tris-HCL (pH8.4), 50mM KCL, 7.5mM $MgCl_2$ (Invitrogen), 1.5 mM of each dNTP (Invitrogen), 5.3ng/µl of random heximer primer pdN6 (Pharmacia Biotech), 16units of RNasin (Promega), and 200units Moloney Murine Leukaemia Virus Reverse Transcriptase (Invitrogen). The reaction was incubated at room temperature for 10 minutes, 37^0C for 50 minutes, 95^0C for five minutes and quenched on ice, cDNA was then stored at -20^0C.

2.3.4. Primer Design

Primers were designed using sequences available from the GenBank database available at (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). OLIGO primer analysis software was used to assess the TM and GC content, mispriming elsewhere in the hMPV genome, and hairpin or primer duplexes. Primers were checked against all other sequences using the

NIBSC Basic Local Alignment Search Tool available at (http://www.ncbi.nlm.nih.gov/BLAST/) for potential cross reactivity with other genomic templates.

2.3.5. PCR Optimisation

All optimisation steps were performed changing only one parameter at a time. A number of different primers were tried in different combinations. A range of buffer conditions were tested (see below). Experiments varying the range of the annealing temperature were carried out on PTC – 225 gradient thermocycler (MJ Research).

2.3.6. PCR Optimisation with the Stratagene Optiprime kit

Five µl of each buffer labelled 1-12 (Table 2.1) were aliquoted in to correspondingly labelled tubes. The following master mix was then made: 12.5µl or Optiprime master mix, 12.5µl of dNTP mix (at 10mM each dNTP), 12.5µl of each primer (50pmol/µl), 125ul of cDNA, 1.5U *Taq* Polymerase. Forty five µl of this mix was added to the 5µl of each buffer. The tubes were incubated at 94^0C for 1 minute followed by 35 cycles of 94^0C for 1 minute, 50^0C for 1 minute, 72^0C for 1 minute. Two µl of the primary round product was added to another 5µl of each corresponding buffer with 43µl of the master mix, as above, and incubated as before.

Table 2.1: Composition of Stratagene PCR optimisation buffers

Buffer Number	Tris –HCL	$MgCL_2$	KCL
1	100mM pH 8.3	15mM	250mM
2	100mM pH 8.3	15mM	750mM
3	100mM pH 8.3	35mM	250mM
4	100mM pH 8.3	35mM	750mM
5	100mM pH 8.8	15mM	250mM
6	100mM pH 8.8	15mM	750mM
7	100mM pH 8.8	35mM	250mM
8	100mM pH 8.8	35mM	750mM
9	100mM pH 9.2	15mM	250mM
10	100mM pH 9.2	15mM	750mM
11	100mM pH 9.2	35mM	250mM
12	100mM pH 9.2	35mM	750mM

2.3.7. Agarose Gel Electrophoresis

For PCR products 15µl was mixed with 2µl of Blue Juice (Invitrogen) and run on a 1.25% MP Agarose gel (Roche Diagnostics) in 1 x TBE (Invitrogen). The amplicons were visualised using ethidium bromide at 5mg/l (Invitrogen) and photographed using the Gel Doc 2000 System (BioRad).

2.3.8. hMPV F Gene PCR 2

The primers used are shown in Table 2.9 and were used at 50pmol. For the primary round PCR 5µl of cDNA was added to 40µl of the reaction mix containing 1x Optiprime Buffer 10 (10mM Tris-HCL, 1.5mM $MgCl_2$ and 75mM KCl) and 2units Taq DNA polymerase (Invitrogen). Amplification using PTC–225 thermocycler (MJ Research) consisted of 1 cycle at 94^0C for 2 minutes followed by 40 cycles of 94^0C for 1 minute, 58^0C for 1 minute, 72^0C for 1 minute. Two µl of primary round product was added to the secondary round amplification mix, as described above with a final concentration of 0.2mM of each dNTP. Amplification consisted of 1 cycle at 94^0C for 2 minutes followed by 35 cycles of 94^0C for 1 minute, 52^0C for 1 minute, 72^0C for 1 minute.

2.3.9. HMPV Attachment Glycoprotein Gene Amplification PCR for Sequencing

The primers used are shown in Table 2.5 and were used at 25pmol. For the primary round PCR 5µl of cDNA was added to 20µl of the reaction mix containing 1x High Fidelity Buffer (60mM Tris-HCL, and 18mM Ammonium Sulphate) 0.2mM $MgCl_2$, 0.2mM of each dNTP and 1U Platinum Taq DNA polymerase High Fidelity (Invitrogen). Amplification using PTC–225 thermocycler (MJ Research) consisted of 1 cycle at 95^0C for 2 minutes followed by 40 cycles of 95^0C for 30 seconds, 50^0C for 30 seconds, 72^0C for 30 seconds followed by and extension of 72^0C for 10 minutes. Two µl of primary round product was added to 48µl of the secondary round amplification mix, as described above. Amplification consisted of 1 cycle at 95^0C for 2 minutes followed by 40 cycles of 95^0C for 30 seconds, 55^0C for 30 seconds, 72^0C for 30 seconds followed by and extension of 72^0C for 10 minutes. 15µl of the second round product was analysed on a gel. If positive 30µl of the remaining product was rerun an a second gel for Gel purification as described below.

2.3.10. HMPV Nucleocapsid and Attachment Glycoprotein/ protein fragments Amplification for Cloning

The hMPV nucleocapsid and attachment glycoprotein gene or gene fragments were amplified by PCR from an hMPV positive clinical sample (Subtype A- by sequencing as described below). Twenty µl of cDNA (as described above) was added to 80µl reaction mix containing a final concentration of 20mM Tris-HCL (pH8.4), 50mM KCl, and 1mM $MgCl_2$ and 3.6 units of taq polymerase (Invitrogen). The primers used are shown in Table 2.6 and 2.7 and were used at 50pmol. Amplification using PTC–225 thermocycler (MJ Research) consisted of 1 cycle at 94^0C for 2 minutes followed by 35 cycles of 94^0C for 1 minute, 51^0C for 1 minute, 72^0C for 1 minute. Amplicons were analysed by agarose gel electrophoresis as described above, and gel purified prior to cloning, as described below.

2.3.11. Gel purification

Gel Fragments were excised from the agarose gel using a clean scalpel. DNA was purified from the agarose using QIAquick gel extraction kit (Qiagen) according to the manufacturers instructions.

2.3.12. Addition of 3' Deoxyadenosine Residues to Proof Reading *Taq* Polymerase Generated PCR Products.

10µl of the gel purified product was added to 10µl reaction mix to give the final concentrations 0.2mM dATP, 20mM Tris-HCL (pH8.4), 50mM KCL, 7.5mM $MgCl_2$, 1unit Taq DNA polymerase (Invitrogen). Samples were incubated at 72^0C for 10 minutes.

2.3.13. Purification of PCR Products

PCR Products were purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturers instruction.

2.3.14. ABI Sequencing

The hMPV G ORF was sequenced using 3.2 pmol of primers indicated in Table 2.5. Using the ABI Big dye Terminator v3.1 Cycling sequencing kit (Applied Biosystems) according to the manufacturers instructions. Termination of the sequencing reaction was achieved by adding 2µl of 125mM EDTA (Sigma) and 3ul of 3M sodium acetate (Sigma) to each

reaction. To clean up the reaction 50µl of ice cold 100% ethanol (VWR international) was added and incubated at room temperature for 15 minutes. Samples were spun at 4^0C 13000rpm on a chilled microcentrifuge for 45 minutes. Ethanol was removed and the pellet washed with 70µl of 70% ice cold ethanol. Excess ethanol was removed and the sample dried using a Savant DNA aped vac 120. Samples were analysed on the Applied Biosystems 377 DNA Sequencer XL (DBS genomics, University of Durham).

2.3.15. Beckman Coulter Sequencing

The partial F gene was sequenced using 3.2 pmol of primer indicated in Table 2.9. Using the Beckman Coulter CEQ 2000 Dye Terminator Cycling sequencing with Quick Start kit (Beckman Coulter) according to the manufacturers instructions. Termination of the sequencing reaction was achieved by adding 2µl of 100mM EDTA (Sigma), 2µl of 3M sodium acetate (Sigma) and 1µl or 20 mg/ml glycogen (supplied with the kit) to each reaction. To clean up the reaction 60ul of ice cold 100% ethanol (VWR international) was added and spun at 4^0C 13000rpm for 15 minutes. Ethanol was removed and the pellet washed twice more with 70µl of 70% ice cold ethanol. Excess ethanol was removed and the sample dried using a Savant DNA aped vac 120. Samples were re-suspended in 40µl of sample loading solution supplied with the kit. Samples were analysed on a Beckman Coulter CEQ 8000 Genetic Analysis System.

2.3.16. Sequence Analysis

Output files from the sequencer were edited to remove questionable data from the ends of the sequences and exported. Files were imported in to SeqMan II (Lasergene, DNAStar) to compare forward, reverse and overlapping sequences to generate a consensus sequence. Consensus sequences we imported in to MegAlign (Lasergene, DNAStar) where they were placed in frame and aligned using the clustal W method for both nucleotide and amino acid sequences.

2.3.17. Sequence and Phylogenetic Analysis

Amino acid alignments of the different data sets were created using Clustal X, and applied to CODON Align with the corresponding nucleotide sequences in order to create in-frame nucleotide alignments. The in-frame nucleotide alignments were subsequently used for the generation of maximum likelihood trees using the program PAUP (4.10b). The criterion

used for creating the trees was determined using the MODELTEST 3.7 (Posanda and Crandall 1998) application within PAUP. Bootstrapping of maximum likelihood trees was impractical due to the large amount of computing time required. Maximum likelihood trees were therefore compared with bootstrapped neighbour joining trees. Where trees were congruent bootstraps are shown on the maximum likelihood trees.

In-frame nucleotide alignments were used to calculate Transition: Transversion Ratios using the program PUZZLE, and Non-synonymous: Synonymous Ratios using the program MEGA 3.1.

2.3.18. Synthesis of hMPV F gene RNA transcripts for analysis of hMPV F Gene RT-PCR 2 sensitivity.

RNA transcripts were prepared from the PTM1 vector containing the hMPV F gene PCR 2 target sequence cloned in frame with a T7 RNA polymerase promoter. The purified plasmid containing the insert was provided by Lucy Beasley and Dr Alison Bermingham, HPA Colindale.

Prior to RNA synthesis the vector was linearised at the unique SmaI restriction site located downstream of the insert using the SmaI restriction enzyme (NEB) according to the manufacturers instructions. The linearised plasmid was analysed by DNA gel electrophoresis and gel purified using the QIAquick gel purification kit (Qiagen) according to the manufacturers instruction.

To generate the RNA transcripts, T7 RNA polymerase (Invitrogen) was used according to the manufacturers instructions. Briefly; 0.1µg of linearised plasmid was mixed with 1µl (50Units) of T7 RNA polymerase, 2µl of 5x T7 reaction buffer, 0.5µl of 0.1M DTT, and 1µl of a 4mM UTP, ATP, CTP and GTP mix. The reaction was made up to a final volume of 10µl with nuclease free water, and incubated at 37 °C for 10 minutes.

Plasmid DNA was removed by RNAse free DNase digest using the Turbo DNA free Kit (Ambion) according to the manufacturers instruction. Briefly; 1µl (2 Units) of turbo DNase was added to 10µl of the transcribed RNA, 5µl of the 10x Turbo DNase buffer and 34µl of nuclease free water. The reaction was incubated for 20 minutes at 37°C. A further 1µl (2 Units) of turbo DNase was added and incubated as before. 10µl of the re-suspended DNase

inactivation reagent was added and incubated at room temperature for 2 minutes. The reaction was centrifuged at 10,000g for 3 minutes and the supernatant containing the RNA was transferred to a fresh tube.

To further ensure full removal of plasmid DNA and the purity of the RNA the RNA was then purified using an RNeasy column (Qiagen) according to the manufacturers instruction and eluted in 30µl of nuclease free water.

The concentration of RNA was measured using a spectrophotometer, and the copy number calculated using the following equation:

$$\text{Copies/ml of RNA} = \frac{\text{Concentration of RNA (g/ml)x Avogadro's number}}{\text{(Size of RNA transcript (bases))(330 x 1nt/bp)}}$$

Where Avogadro's number = 6.022×10^{23}

2.4. Cloning Techniques

2.4.1. Bacterial Cell Culture and Medium

Transformed *E.coli* were grown on Luria-Bertani Agar (LB, 1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, 0.1% glucose, 1% difco agar in distilled water) plates containing 100μg/ml ampicillin or 50μg/ml kanamycin where appropriate and incubated at 37^0C overnight.

Lurina-Bertani Broth (same as above without the 1% difco agar) containing 100μg/ml ampicillin or 50μg/ml kanamycin where appropriate and incubated at 37^0C overnight on an orbital shaker at 200rpm.

All media reagents were made in the Colindale HPA media department, and were autoclaved at 15psi and 120^0C for 20 minutes.

2.4.2. TOPO Cloning

Gel extracted or PCR purified PCR products (with 3' end deoxyadenosine residues) were cloned in to the TOPO TA pCR 2.1-TOPO cloning vector (Invitrogen) according to the manufacturers instruction.

2.4.3. Ligation in to other Vectors Using Restriction Sites

Different ratios of vector DNA: insert DNA (3:1, 1:1, 1:3 and 1:10, where 100ng of vector DNA was used in the 1:1 ratio) were set up in 20μl reaction volumes containing 400units of T4 DNA Ligase (New England Biolabs) and 1x T4 DNA ligase Buffer (50mM Tris-HCL (pH7.5), 10mM $MgCl_2$ 10mM dithiothreitol, 1mM ATP, 25μg/ml bovine serum albumin) and incubated overnight at 16^0C. The ligation mix was transformed in to *E.coli* as described below.

2.4.4. Transformation of *E.coli*

Two μl of plasmid DNA or Ligation mix were transformed in to One Shot Chemical competent Top 10 *E.coli* by heat shock according to the manufactures instructions (Invitrogen). 250μl of SOC medium was added and incubated at 37^0C for 1 hour on an orbital shaker at 200rpm. Cells were plated on to LB Plates containing the appropriate

selective antibiotic for the plasmid, as described above and pre-treated with 40µl of 40 mg/ml X-gal (Invitrogen) where appropriate.

2.4.5. Small Scale Purification of Plasmid DNA

Cells containing the plasmid of interest were pelleted from a 2ml culture using a microcentifuge at 13000g and the plasmid DNA purified using the Qiaprep Spin Miniprep kit (Qiagen) according to the manufacturers instructions.

2.4.6. Large Scale Purification of Plasmid DNA

A 5 ml miniprep culture of *E.coli* containing the plasmid of interest with the appropriate selective antibiotic was incubated for 8 hours. A 1:200 dilution of the miniprep culture was made to give a final 500ml volume with the appropriate selective antibiotic and was incubated over night at 37^0C on an orbital shaker at 200rpm. Cells were pelleted by centrifigation at 6000rpm, and the media discarded. Plasmid DNA was purified using an Endofree Plasmid Maxi Kit (Qiagen).

2.4.7. Restriction Digest Analysis

Restriction digests were carried out using enzymes with their supplied buffers at a 1x concentration (New England Biolabs) according to the manufacturers instruction. Usually 1 unit of enzyme for every 1µg of DNA in a 30ul reaction mix. Where double digests were carried out the optimal buffer was selected according to the manufacturers guidelines.

2.5. Recombinant Baculovirus and Insect Cell Culture Techniques

2.5.1. Insect Cell Culture

SF9 cells (Table 2.2) were maintained every 3 to 4 days in SF–900 II medium with L-glutamine (Invitrogen) and supplemented to a final concentration of 2% FBS (Invitrogen) and 50µg/ml Gentamicin (Invitrogen). Cells were seeded at $5x10^5$ cells/ml in a 50ml volume in a 250ml Corning Erlenmyer flask (Sigma), and passaged every 3 to 4 days, not allowing the cells to reach densities above $1X10^7$ cells/ ml.

High 5 cells (Table 2.2) were maintained as above every 2 –3 days using serum free Express 5 medium (Invitrogen) supplemented with 0.2mM L-glutamine (Invitrogen).

2.5.2. Preparation of Bacmid DNA

20µg of purified Bacmid DNA (kindly supplied by Prof. Ian Jones, University of Reading) was linearised by restiction digest with BSU361, (for which there is a unique site within the bacmid DNA) with 1x NEB Digest buffer3 (50mM Tris-HCL, 100mM Nacl, 10mM $MgCL_2$, 1mM dithiothreitol, pH 7.9) with 1.2ng/µl BSA and 60Units BSU 361 restriction endonuclease (New England Biolabs) in a total reaction volume of 80ul. The reaction was incubated 37^0C for two hours followed by the addition of another 6µl of BSU 361 and incubate for a further 2 hours at $37\ ^0C$ and inactivated at 80^0C for 20 minutes.

2.5.3. Generation of Recombinant Baculovirus

A 6 well tissue culture plate was seeded with Sf9 cells at 1 x 10^6 cells/well in 2ml of growth medium and allowed to adhere for 30 minutes at room temperature. The growth medium was removed and the cells washed once and replace with serum and antibiotic free SF–900 II medium

The transfection mix was made containing 1µg of Bacmid DNA, 0.5µg of Transfer vector DNA containing insert, 8µl of Reagent (Invitrogen) and made up to a final volume of 24µl with Nuclease free water (Promega) and incubated at room temperature for 15 minutes. One ml of serum/ antibiotic free medium was added to the mixture. The entire transfection mixture replaced the medium on the cells and incubated for 16 hours at 28°C. The transfection mixture was replaced with 2 ml of growth medium and incubated for a further 5 days. Cells were monitored daily for CPE.

2.5.4. Recombinant Baculovirus Growth

Five ml of SF9 cells were seeded at 8 x 10^6 cells /ml, and incubated for 2 hours at 28^0C with 1ml of the transfection mix or 0.1MOI of recombinant baculovirus for preparation of high titred virus stock. The volume was made up to 50 ml with growth medium and incubate at 28^0C shaking at 115rpm for 4 days. Cells were pelleted at 1000RCF for 15 minutes and the supernatant containing the virus harvested. For protein production the procedure was repeated as above using 3x10^7 cells /ml in 5 ml for seeding and 3MOI or more of recombinant baculovirus. These cells were collected by centrifugation as above and prepared for protein purification.

When using High 5 cells for protein production the procedure was repeated as above using 5ml of 1x 10^7 cells/ml for seeding with 5MOI of virus.

2.5.5. Plaque Assay

Six well tissue culture plate was seeded with Sf9 cells at 1 x 10^6 cells/well in 2ml of growth medium and allowed to adhere for 30 minutes at room temperature. Media was removed and replaced with 1ml of fresh growth medium. Ten fold serial dilutions (10^{-1} to 10^{-8}) of the virus was made in 1ml of growth medium and 100µl of the diluted virus staring with the 10^{-3} dilution were added to the 1ml of media in duplicate wells, to give final dilution range of 10^{-4} to 10^{-9} of the virus. Virus was absorbed for 2 hours at 28^0C. Media was removed and cells overlaid with 2ml of 3.2% Agarose type VII (Sigma) mixed 1:1 with growth medium, to give a final concentration of 1.6% agarose. Once set. 1 ml of growth medium was added on top of the agarose. Cells were incubated at 28^0C for 6 days. Plates were stained with 2ml of Neutral Red (Sigma) diluted 1:20 with PBS (final concentration of 165mg/l of neutral red) for 2 hours. The stain was removes and plates dried for 6 hours before the plaques were counted.

2.6. Analysis of Expressed Baculovirus Proteins

2.6.1. SDS PAGE gel

Protein samples were resolved by discontinuous SDS polyacrylamide gel electrophoresis (SDS PAGE). Prior to analysis cells were lysed with the appropriate lysis buffer; 1%(v/v) nonident P-40 for mammalian cells or 1x Insect pop culture reagent for insect cells (Novagen). The required amount of sample was mixed to give a final concentration of 1x Nupage LDS sample buffer (Invitrogen) and 1x Nupage Sample reducing agent (Invitrogen) containing DTT and heated for 10 minutes at 100^0C. The samples (15-25µl) were run on 10 or 15 well 4-12% Nupage Novex Bis-Tris acrylamide gels(1.5mm thick) (Invitrogen), for approximately 1 hour at 180V with 1 x MOPS or 1 x MES Buffer (Invitrogen) using the XCell SureLock Mini Cell electrophoresis equipment (Invitrogen).

2.6.2. Western Blotting

Proteins were transferred from SDS PAGE gel on to Hybond nitrocellulose ECL membranes (Amersham Biosiences) using the XCell SureLock Mini Cell transfer equipment (Invitrogen), with 1x Nupage transfer buffer (Invitrogen) and 10% Methanol per gel/membrane transfer. After transfer membranes were blocked for 1 hour at room temperature or overnight at 4^0C in 25ml PBS containing 5% (w/v) dried skimmed milk powder. Membranes were then incubated with primary antibody diluted in PBS containing 0.1% (v/v) TWEEN 20 (VWR International) and 5% (w/v) dried skimmed milk powder for 1 hour at room temperature. Membranes were washed 3 times for 10 minutes with PBS containing 0.1% (v/v) TWEEN 20. Membranes were incubated with secondary HRP conjugated antibody diluted in PBS containing 0.1% (v/v) TWEEN 20 and 5% (w/v) dried skimmed milk powder for 1 hour at room temperature. Membranes were washed 3 times for 10 minutes with PBS containing 0.1% (v/v) TWEEN 20. Bound antibody was detected using the chemoluminescence (ECL) detection reagents (Amersham Biosciences). Membranes were wrapped and exposed to Hyperfilm ECL (Amersham Biosciences) for 1-20 minutes. Film was developed manually using CD18 Developer and fixer (GRI limited.)

2.6.3. Coomassie Stain

Proteins run on SDS PAGE gels were visualised using Simply Blue Coomassie safe stain (Invitrogen). The gel was washed 3 times for 5 minutes with distilled water. Enough Safe

stain was used to cover the gel, and was incubated on a rocker for 1hour at room temperature. The gel was washed 3 times for 5 minutes with distilled water, and incubated in distilled water on a shaker overnight to destain. Gels were preserved using a gel drying solution Kit (Invitrogen) according to the manufacturers instructions.

2.6.4. BioRad protein assay

To quantitate total protein by a protein assay. The BioRad DC Protein assay kit was used to the manufacturers instruction.

2.6.5. Immunofluorescence

Transfected or infected cells were fixed by 80% ice cold acetone in PBS or 4% paraformaldehyde for 10 minutes. Cells fixed with paraformaldehyde were permeabilised with 0.25% Triton X-100 (v/v) and washed 3 times for 5 minutes with PBS containing 0.1% (v/v) TWEEN 20. Cells were blocked with 1% BSA (v/v) in PBS for 15 minutes, and washed as before. Cells were incubated with primary antibody diluted in PBS containing 0.1% (v/v) TWEEN 20 and 1% BSA (v/v) for 30 minutes at 37^0C. Cells were washed as before. Cells were incubated with Secondary FITC conjugated antibody diluted in PBS containing 0.1% (v/v) TWEEN 20 and 1% BSA (v/v) for 30 minutes at 37^0C. In some instances 0.01% Evans blu counterstain was included in the secondary antibody diluent. Cells were washed as before. In some instances 1ug/ml DAPI Nuclear Stain(Roche) was incubated with the cells for 15minues at room temperature, and washed as before. Fluorescence was analysed using a Zeiss Axiovert 200 Inverted Microscope. Images were captured with a Zeiss AxioCam HRc and Axio vision software.

2.7. Protein Purification

2.7.1. Preparation of cell lysate

SF9 cells infected with recombinant baculovirus expressing the hMPV N protein were pelleted by centrifugation at 3000rpm for 10 minutes. The cells were resuspended in one fifth of the original culture volume with 1x Insect pop culture reagent for insect cells (Novagen) supplemented with 1x protease inhibitor cocktail set I (CalBiochem) and 10units/ml of Benzonase Nuclease (Novagen) and incubated at room temperature for 15 minutes. The cell lysate was clarified by centrifugation at 3000rpm for 10 minutes. The clarified supernatant was purified by Ni-NTA affinity column, or stored at -20^0C.

SF9 cells infected with recombinant baculovirus expressing the hMPV G-FL protein were pelleted by centrifugation at 3000rpm for 10 minutes.

The cells were lysed by resuspending the pellet in either:

- 1x Insect pop culture reagent
- 1% NP-40
- 0.1% Triton X-100
- 2% Tween-20

Each lysis reagent was supplemented with 1x protease inhibitor cocktail set I (CalBiochem) and 10units/ml of Benzonase Nuclease (Novagen) and incubated at room temperature for 15 minutes. The cell lysate was clarified by centrifugation at 3000rpm for 10 minutes.

2.7.2. Ni NTA protein purification of the hMPV N protein

The 6 x histadine Tagged hMPV N protein was purified using a Ni-NTA his bind resin and buffer kit (Novagen) under native conditions according to the manufacturers instructions. Purification required some optimisation of buffer conditions.

Briefly; the lysed cell preparation (described in 2.7.1) was clarified by centrifugation and applied to the Ni-NTA bind slurry and incubated on a shaker at 4°C for 2 hours. The slurry contained Ni-NTA sepharose beads and 10mM imidazole buffer. The mixture was then packed in to a column for the washing and elution steps. Unbound and non-specific proteins were removed using increasing concentrations of imidazole buffer. N-FL was

eluted using a 250mM imidazole buffer. Each of the fractions was collected and analysed by ELISA, Western blot and protein gel for purity of the purified eluted protein fragments, as described in section 2.6.

2.8. Generation of hMPV Specific Antiserum

HMPV specific antiserum was raised against whole virus grown in cell culture, and baculovirus expressed recombinant hMPV nucleocapsid protein and attachment glycoprotein using female New Zealand White Rabbits. Summarised details of the antigen, prepared innoculum and inoculation schedule for each antiserum is provided in Table 2.2. An schematic of the inoculation and bleeding schedule for each antiserum is shown in Figure 2.1 below.

Table 2.2: hMPV Specific antiserum raised and evaluated in this study.

Antiserum	Immunogen
Rabbit α-hMPV Total	hMPV infected RMK cell lysate approximately $1x10^5$ cells/ml. Unknown amount of hMPV virus, assumed to be very low titre, no adjuvant was used. 4 x 0.25ml of the inoculum was injected at 4 points along the dorsal of the rabbit with a booster given on day 30 into the hind leg muscle. Terminal Bleed taken day 60.
Rabbit α-hMPV N-FL	**Purified baculovirus expressed hMPV N-FL** **100ng of purified protein 10mM Purified LPS 0.5ml incomplete Freund's adjuvant. 4 x 0.25ml of the inoculum was injected at 4 points along the dorsal of the rabbit.100ng purified N-FL with 10mM LPS and saline was given as a second immunogen on days 21 and 40 into the hind leg muscle. Test bleeds taken days 35 and 55. Terminal Bleed taken day 60.**
Rabbit α-hMPV G (s)-Fc	Baculovirus expressed hMPV G(s)-Fc High 5 supernatant 100ng of total protein from the cell supernatant with 10mM Purified LPS 0.5ml incomplete Freund's adjuvant. 4 x 0.25ml of the inoculum was injected at 4 points along the dorsal of the rabbit. 100ng of total protein from the cell supernatant with 10mM LPS and saline was given as a second and third immunogen on days 21 and 40 into the hind leg muscle. Test bleeds taken days 35 and 55. Terminal Bleed taken day 60.

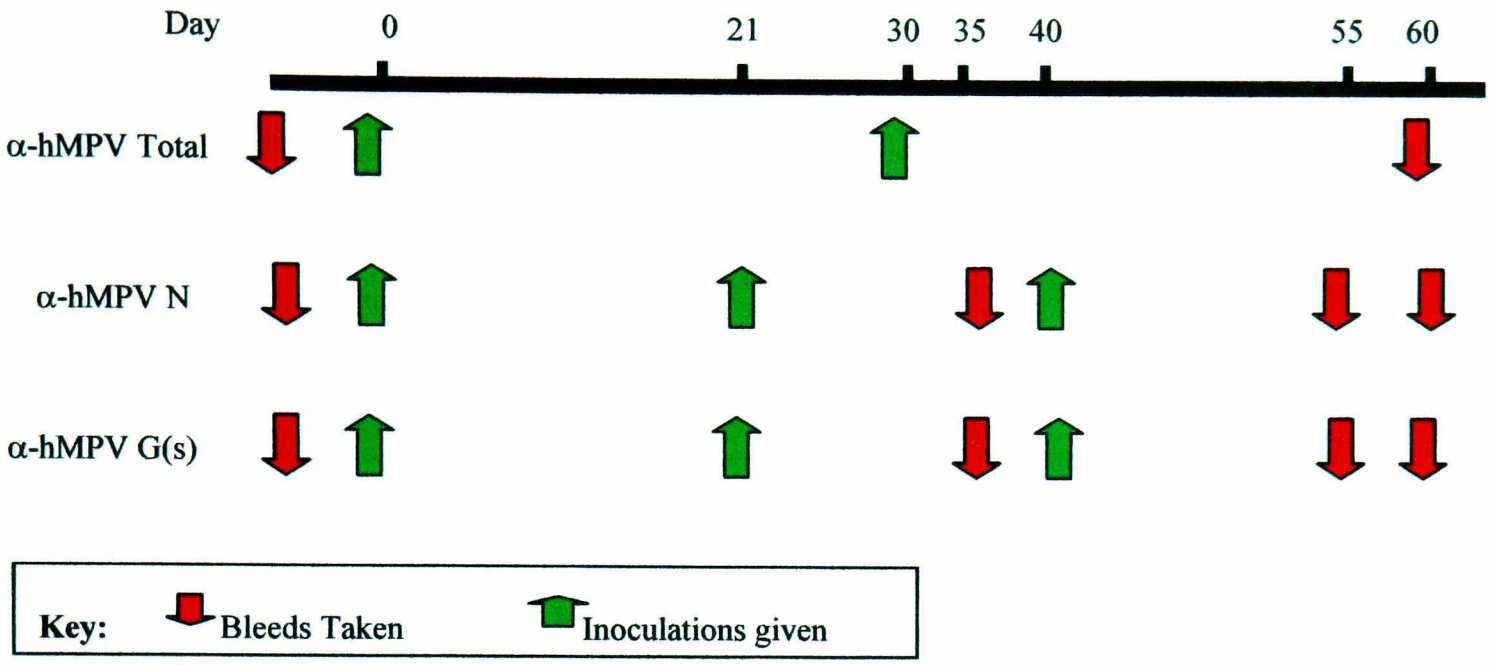


Figure 2.1: Schedule of rabbit inoculations for the generation of hMPV specifc antiserum. **(i)** Rabbit α-hMPV Total was generated by Dr Joanne Stockton at the HPA Colindale. **(ii)** hMPV N-FL Specific polyclonal serum was raised against 100ng of hMPV N-FL mixed with purified lipopolysaccharide and incomplete Freund's adjuvant to form an emulsion. **(iii)** hMPV G(s) Specific polyclonal serum was raised against 100ng of hMPV G(s) Supernatant mixed with purified lipopolysaccharide and incomplete Freund's adjuvant to form an emulsion.

2.8.1. Rabbit α-hMPV total

The Rabbit α-hMPV total antiserum was prepared and provided by Dr Joanne Stockton, HPA Colindale. hMPV was isolated from a combined nose and throat swab collected in 2001 as part of the Leicester study described in Chapter 3 and is an A subgroup virus but is a different strain to those used elsewhere in the study. The details describing the isolation of this strain are unavailable. The inoculum used to generate this rabbit serum is an hMPV infected RMK cell lysate of approximately $1x10^5$ cells/ml. The lysate was prepared by lysis with distilled water, sonicated for 15 minutes and diluted 1:2 with saline. 4 x 0.25ml of the inoculum was injected at 4 points along the dorsal of the rabbit with a booster given on day 30 into the hind leg muscle. The terminal bleed was taken day 60, and the serum cross absorbed against RMK cells to remove antibody directed towards cellular proteins.

2.8.2. Rabbit α-hMPV N

For the hMPV nucleocapsid protein the primary inoculation consisted of 100ng of purified N protein (For expression purification methodology of hMPV N see sections 2.5, 2.6 and 2.7 and Chapter 5 for more detail) , with 10mM purified lipopolysaccharide and incomplete freunds adjuvant kindly supplied by Dr Henrick Chart to total volume of 1ml and sonicated to form an emulsion. Four x 0.25ml of the emulsion was injected at 4 points along the dorsal of the rabbit. The following 2 booster inoculations given at 21 and 40 days contained 100ng of purified N and 10mM purified lipopolysaccharide. Saline was used to make the volume up to 1ml. 0.5 ml was injected in to each of the hind legs. Two test bleeds were taken on days 33 and 55. Terminal bleed was taken day 62.

2.8.3. Rabbit α-hMPV G(s)-Fc

The rabbit α-hMPV G(s)-Fc was raised against baculovirus expressed recombinant hMPV attachment glycoprotein High 5 cell culture supernatant. Cell culture supernatant containing 100ng of total protein was mixed with 10mM Purified LPS 0.5ml incomplete Freund's adjuvant kindly supplied by Dr Henrick Chart to total volume of 1ml and sonicated. 4 x 0.25ml of the inoculum was injected at 4 points along the dorsal of the rabbit. Two booster inoculations of the baculovirus expressed hMPV G(s)-Fc cell culture supernatant containing 100ng of total protein mixed with 10mM Purified LPS and made up to 1ml with saline were given on days 21 and 40 into the hind leg muscle. Test bleeds taken days 35 and 55. Terminal Bleed taken day 60. The serum cross absorbed against High 5 cells to remove antibody directed towards cellular proteins.

2.9. ELISA Techniques

2.9.1. ELISA Optimisation

All optimisation steps were performed changing only one parameter at a time. A number of different coating buffers, antigen, primary and secondary antibody formulas and concentrations were tested as described in chapter 6.

2.9.2. Antigen Preparation for ELISA

Uninfected and hMPV N baculovirus infected cell lysate was prepared in one batch to provide enough antigen for the study. SF9 cells infected with recombinant baculovirus expressing the hMPV N protein were pelleted by centrifugation at 3000rpm for 10 minutes. The cells were resuspended in one fifth of the original culture volume with 1x Insect pop culture reagent for insect cells (Novagen) supplemented with 1x protease inhibitor cocktail set I (CalBiochem) and 10units/ml of Benzonase Nuclease (Novagen) and incubated at room temperature for 15 minutes. The cell lysate was clarified by centrifugation at 3000rpm for 10 minutes. Nucleoprotein positive and uninfected cell lysate were standardised to contain the same amount of total protein by gel analysis.

2.9.3. Serological ELISA for the Detection of Human IgG Against hMPV Nucleoprotein.

A 96 well U bottom Nunc Maxisorb ELISA plate (Fisher scientific) was coated overnight at 4^0C with 100µl per well of hMPV N positive Sf9 cell lysate or uninfected cell lysate (as described above) diluted 1:200 with 20mMTris HCL (pH9.6) to give a final total protein concentration of 400ng/ well. Plates were washed 4x with PBS containing 0.05% TWEEN 20 (v/v) using a Tecan Plate washer. Plates were incubated with 100µl of test or control serum diluted 1:100 with PBS containing 0.5% (v/v) TWEEN 20 and 5% (w/v) dried skimmed milk powder for 1 hour at 37^0C on an orbital Plate shaker (Edmund Bühler TiMix) at 600rpm. Each test serum or control serum was tested in duplicate against both the hMPV N SF9 cell lysate and uninfected cell lysate. Plates were washed as before and incubated with 100µl of Rabbit α- Human IgG HRP conjugated antibody (Dako) at 1:5000 dilution with PBS containing 0.5% (v/v) TWEEN 20 and 5% (w/v) dried skimmed milk powder and incubated as before. Plates were washed as before and incubated in the dark with 100µl of TMB substrate (Europa Bioproducts Ltd M0701A) for 5 minutes. The

reaction was stopped with 100µl of 0.5M HCL (VWR International). The Absorbance at 450nm (reference 670nm) was measured using a 96 well Tecan Spectra Image plate reader.

2.9.4. Correction and Data analysis

Subtract the OD 450 value for the hMPV N negative cell lysate was subtracted from that of the N positive cell lysate to give the corrected OD450 Value. Each sample was tested in duplicate so the average corrected OD450 value was determined. Samples with an average corrected OD450 value below the OD450 Cut off value of 0.4 were negative for hMPV N IgG, while those at or above were positive.

2.9.5. Statistical analysis

Where appropriate statistical analysis was performed using the students t-Test.

2.10. Transient expression of the full length hMPV G protein in mammalian cells.

2.10.1. Transfection

The purified pTriex vector containing the full length hMPV G gene was transfected into 293T / Vero cells using Lipofectamine 2000 (Invitrogen) according to the manufacturers instruction.

96 well tissue culture plates were seeded with $5x10^4$ cells /well of 293T or vero cells in growth medium [Minimum Essential Medium (MEM) (1X) liquid containing Earle's salts, and 25 mM HEPES (Invitrogen) supplemented with a final concentration of 10% Foetal Bovine Serum (Invitrogen), 2mM Glutamax I (Invitrogen), 50µg/ml Gentamicin (Invitrogen), and 1x MEM Non- Essential amino acid solution (Sigma)]. And incubated overnight , or until 90% confluent monolayer's were formed, in a 37°c incubator with 5% CO_2. On the day of transfection the growth medium was removed and the cells washed three times with 100µl of transfection medium [Minimum Essential Medium (MEM) (1X) liquid containing Earle's salts, and 25 mM HEPES (Invitrogen) supplemented with a final concentration of 2mM Glutamax I (Invitrogen) and 1x MEM Non- Essential amino acid solution (Sigma)]. After washing 100µl of transfection medium was dispensed per well.

The transfection mix was prepared by diluting 0.2-0.8µg of the plasmid DNA (0.4µg was optimal) in 25µl of transfection medium. In a separate tube 0.5µl of the Lippofectamine 2000 reagent was diluted in 25 µl of transfection media and incubated at room temperature for 5 minutes. The diluted transfection reagent was the added to the diluted plasmid and incubated at room termperature for a further 20 minutes. The entire 50µl of the transfection mixture was then added to each well of the 96 well plate containing the cells and 100 µl of the transfection media. The plate was incubated in a 37°c incubator with 5% CO_2 for 24-72 hours. For those plates being incubated > 24 hours, after the initial 24 hours the media and transfection reagent were removed, and replaced with 100µl of fresh growth medium.

2.10.2. Immunofluorescence analysis

Transfected cells were fixed with 4% paraformaldehyde for 10 minutes, permeabilised with 0.25% Triton X-100 (v/v) and washed 3 times for 5 minutes with PBS containing 0.1% (v/v) TWEEN 20. Cells were blocked with 1% BSA (v/v) in PBS for 15 minutes, and washed as before. Cells were incubated with Mouse α-6x histidine MAb diluted 1:50 in PBS containing 0.1% (v/v) TWEEN 20 and 1% BSA (v/v) for 30 minutes at 37^0C. Cells were washed as before, and incubated with a Rabbit α-mouse IgG FITC conjugated antibody diluted 1:40. 1ug/ml DAPI Nuclear Stain(Roche) was incubated with the cells for 15minues at room temperature, and washed as before. Fluorescence was analysed using a Zeiss Axiovert 200 Inverted Microscope. Images were captured with a Zeiss AxioCam HRc and Axio vision software.

Table 2.3 Cell lines and Bacterial Strains used in this study

Cell Line	Comments	Source
LLC-MK2	Rhesus Monkey Kidney Epithelial Cells	ATCC
293T	Human Kidney Epithelial cells transformed with adenovirus 5 DNA, expressing T7 promoter	ATCC
Hep- 2	HeLa contaminant – Human Adenocarcinoma Cervix Epithelial Cells	ATCC
HEP2 V	Hep-2 cells stably expressing SV-5 V protein.	Prof. Rick Randal University of St Andrews
A549	Human Lung Carcinoma Epithelial Cells	ATCC
BS-C-1	African Green Monkey Kidney Epithelial cells	ATCC
VERO	African Green Monkey Kidney Epithelial Cells	ATCC
RMK	Primary Monkey Kidney Cells	Cambridge HPA
SF9	Insect cell line from *Spodoptera frugiperda*	Invitrogen
HIGH 5	Insect cell line from *Trichoplusia ni*	Invitrogen
TOP 10	Chemically competent *E.coli*	Invitogen

Table 2.4 Vectors and Constructs used and generated in this study

Plasmid	Comments	Antibiotic Resistance	Source
PCR –2.1-TOPO	Linearised vector with 3' – thymidine overhangs for TA cloning, covalently bound to Topoisomerase I	Ampicillin/ Kanamycin	Invitrogen
PTriEx-2 Neo	3 system expression vector with a hybrid CMV chicken β-actin promoter for mammalian cell expression, flanking polh baculovirus sequences for homologous recombinanation and p10 promoter for expression in insect cells, and T7 *lac* promoter for expression in *E.coli*. A number of tags are available.	Ampicillin / Neomycin Sulphate	Novagen
PtriEx – human IgGFc	Same as PTriEx-2 Neo vector engineered to contain a carboxyl terminal human IgG FC for fusion proteins when using the engineered Sfi1 sites.	Amplicillin	Prof. Ian Jones University of Reading
PTriEx-2 Neo – hMPV N ORF	PTriEx-2 Neo vector with hMPV Nucleoprotein ORF subtype A derived from a clinical sample inserted with Not1 Bam H1 sites with in frame amino terminal 6 x Histadine tag.	Ampicillin / Neomycin Sulphate	This Project
PTriEx-2 Neo – hMPV N Fragment A	PTriEx-2 Neo vector with hMPV Nucleoprotein Fragment A inserted with Not1 Bam H1 with in frame amino terminal 6 x Histadine tag.	Ampicillin / Neomycin Sulphate	This Project
PTriEx-2 Neo – hMPV N Fragment B	PTriEx-2 Neo vector with hMPV Nucleoprotein Fragment B inserted with Not1 Bam H1 with in frame amino terminal 6 x Histadine tag.	Ampicillin / Neomycin Sulphate	This Project

Plasmid	Comments	Antibiotic Resistance	Source
PTriEx-2 Neo – hMPV N Fragment C	PTriEx-2 Neo vector with hMPV Nucleoprotein Fragment C inserted with Not1 Bam H1 with in frame amino terminal 6 x Histadine tag.	Ampicillin / Neomycin Sulphate	This Project
PTriEx-2 Neo – hMPV N Fragment D	PTriEx-2 Neo vector with hMPV Nucleoprotein Fragment D inserted with Not1 Bam H1 with in frame amino terminal 6 x Histadine tag.	Ampicillin / Neomycin Sulphate	This Project
PTriEx-2 Neo – hMPV N Fragment F	PTriEx-2 Neo vector with hMPV Nucleoprotein Fragment F inserted with Not1 Bam H1 with in frame amino terminal 6 x Histadine tag.	Ampicillin / Neomycin Sulphate	This Project
PTriEx-2 Neo hMPV G ORF	PTriEx-2 Neo vector with hMPV Attachment Glycoprotein ORF subtype A derived from a clinical sample inserted with Not1 Bam H1 with in frame amino terminal 6 x Histadine tag.	Ampicillin / Neomycin Sulphate	This Project
PtriEx – human IgGFc hMPV G(s)	PtriEx – human IgGFc vector with hMPV truncated Attachment Glycoprotein ORF without the cytoplasmic/ transmembrane regions inserted with Not1 Bam H1 sites with in frame amino terminal 6 x Histadine tag.	Ampicillin	This Project
Bac 10:KO_{1629} (Referred to as Bacmid)	Plasmid encoding the entire genome of AcMNV lacking an essential gene for replication. Used for the generation of recombinant baculovirus when co transfected with pTriEx-2 Neo vectors.	Kanamycin	Prof. Ian Jones University of Reading
PTM1- hMPV F Gene PCR 2 target	PTM1 vector with the hMPV F gene 2 PCR target cloned in frame with a T7 promoter	Ampicillin	L. Beasley HPA Colindale

Table 2.5 Commercial / Non-Clinical Antiserum used in this study

Antiserum	Comments	Source
Mouse anti 6 x histidine	A monoclonal antibody(IgG_1) against five consecutive histidine residues.	Novagen
Rabbit anti mouse Immunoglobulins HRP	Peroxidase conjugated antibody for the detection of Mouse Immunoglobulin primary antiserum for techniques such as ELISA or Western Blot.	DakoCytomation
Rabbit anti Mouse Immunoglobulins FITC	FITC conjugated antibody for the detection of Mouse Immunoglobulin primary antiserum for techniques such as Immunofluorescence	DakoCytomation
Rabbit anti hMPV total	Polyclonal Rabbit antiserum raised against hMPV infected RMK cell lysate	This study
Rabbit anti hMPV N-his	Polyclonal Rabbit antiserum raised against purified baculovirus expressed hMPV nucleoprotein	This study
Rabbit anti hMPV G(s)	Polyclonal Rabbit antiserum raised against baculovirus expressed hMPV G(s) Sf9 cell lysate	This study
Rabbit anti hMPV N-his FITC	FITC conjugated IgG Polyclonal Rabbit antiserum raised against purified baculovirus expressed hMPV nucleoprotein	This study with Dr Dhan Samuels HPA Colindale
Goat anti Rabbit HRP	Peroxidase conjugated antibody for the detection of Rabbit IgG primary antiserum for techniques such as ELISA or Western Blot.	Kirktengaard and Perry Laboratories

Antiserum	Comments	Source
Swine anti Rabbit FITC	FITC conjugated antibody for the detection of Rabbit IgG primary antiserum for techniques such as Immunofluorescence	DakoCytomation
Rabbit anti Human IgG HRP	Peroxidase conjugated antibody for the detection of human IgG primary antiserum for techniques such as ELISA or Western Blot.	DakoCytomation
Rabbit anti Human IgG FITC	FITC conjugated antibody for the detection of human IgG primary antiserum for techniques such as Immunofluorescence	DakoCytomation
Rabbit anti Human IgG Fab2 HRP	Peroxidase conjugated antibody for the detection of human IgG FAB 2 region of primary antiserum for techniques such as ELISA or Western Blot.	Sigma
Human Serum Pool	Adult human serum submitted to the Respiratory Virus Unit, HPA, Colindale as part of routine influenza surveillance. The pool is made from adult sera negative fir influenza IgG in 2001	Respiratory Virus unit, HPA Colindale
Human sera $\leq$ 15 years	Colindale HPA (2001-2004) sera submitted to the HPA for serological investigation of influenza.	Respiratory Virus unit, HPA Colindale
Human sera $\geq$ 15 years	Colindale HPA (2001-2004) sera submitted to the HPA for serological investigation of influenza.	Respiratory Virus unit, HPA Colindale

Adult acute and convalescent RSV A/B sera	Colindale HPA (2001) sera submitted as part of investigation in to the cause of ARTI in particular hRSV in community dwelling elderly > 65 years old (Chapter 3)	Respiratory Virus unit, HPA Colindale
Paediatric acute and convalescent RSV A/B sera	Colindale HPA. Neonatal paediatric sera collected as part of a longitudinal cohort study of RSV infection in childhood (Althani, 2004).	Respiratory Virus unit, HPA Colindale
Age stratified panel of sera ≤ 72 months	Colindale HPA. Sera from UK children submitted to the HPA for investigated of rash illness	Immunisation and Diagnosis Unit, HPA Colindale

Table 2.6 hMPV G Gene Sequencing Primers

Primer	Gene	Position*	Sense	Sequence 5'-3'	Source
HMPVG6100F[a]	G	6100-6120	+	TTTAAAAATATTTTGAAAAC	This study
HMPVLABR[a]	L	7185-7204	-	AGATAGACATTAACAGTGGA	This study
HMPVGAF[a]	G	6233-6259	+	GGGACAAGTAGTTATGGAGGT GAAAG	This study
HMPVGBFa	G	6233-6259	+	GGGACAAGTGGCCATGGAAGT AAGAG	This study
M13F[b]	TOPO		-	GTAAAACGACGGCCAG	Invitrogen
M13R[b]	TOPO		+	CAGGAAACAGCTATGAC	Invitrogen
HMPVGA1 104F[c]	G	6348-6365	+	CCTAATAGGAATAACTAC	This study
HMPVGA1 529R[c]	G	6789-6770	-	GCTGCTTGTGCGGAGAGTGG	This study
HMPVGA1 332F[c]	G	6574-6503	+	GCAAGCTCACCAGAGACAGAA CC	This study
HMPVGA2 126F[d]	G	6367-6387	+	CTGAGTATAGCCCTCAATATC	This study
HMPVGA2 226F[d]	G	6467-6488	+	CAATCCAAACTCACAGCATCC	This study
HMPVGA2 494R[d]	G	6754-6733	-	CCTTCGCTGTTGTCCGTGGTGG	This study
HMPVGB1 126F[e]	G	6367-6384	+	GCATGGCACTTAATATTTTCC	This study
HMPVG B1 519R[e]	G	6773-6757	-	GTTGGGTGCAGCTGTGC	This study

Footnotes: (a) Primers used for amplification of the hMPV G ORF, (b) primers located in the TOTO vector used for the first round of sequencing, (c) Primers used for the second round of sequencing for A1 strains, (d) Primers used for the second round of sequencing of A2 strains, (e) primers used for sequencing of B1 strains, (*) Sequence positions according to reference strain NL-001.

Table 2.7 hMPV G Gene Cloning Primers

Primer	Gene	Position*	Sense	Sequence 5'-3'	Source
HMPVGBAMH1F[a]	G	6247-6267	+	GGATCCTATGGAGGTGAAAG TGGAGAA	This study
HMPVGBAMH1 STOPR[a]	G	6956-6937	-	GGATCCTGTTAACTAGTTTG GTTGTATG	This study
HMPVG (S) BACF[b]	G	6406-6421	+	GGCCATTATGGCCATACAAT GCAAGAAAAC	This study
HMPVG (S) BACR[b]	G	6956-6937	-	GGCCGAGGCGGCCACTAGTT TGGTTGTATGTTGTTGA	This study

Footnotes: (a) Primers used for amplification of G-FL for cloning in to the pTRiEx vector (b) Primers used for amplification of G(s) for cloning in to the pTRiEx vector, (*) Sequence positions according to reference strain NL-001.

Table 2.8 hMPV N Gene Cloning Primers for the pTRiEX vector.

Primer	Gene	Position*	Sense	Sequence 5'-3'	Source
HMPV N PRIMER A PTRIEX	N	40-59	+	GGATCCCATGTCTGTTCA AGGGATTCACC	This study
HMPV N PRIMER B PTRIEX	N	520-502	-	GCGGCCGCCTTAGATTAT GGGTGTGTCTGG	This study
HMPV N PRIMER C PTRIEX	N	520-538	+	GGATCCCTTATTATGTGT AGGTGCC	This study
HMPV N PRIMER D PTRIEX	N	880-859	-	GCGGCCGCCTTAACCTTG GACGGATACATG	This study
HMPV N PRIMER E PTRIEX	N	880-899	+	GGATCCCGAGTTAAAACA GGTCACAG	This study
HMPV N PRIMER F PTRIEX	N	1225-1206	-	GCGGCCGCCTTACTCATA ATCATTTTGAC	This study

Footnotes: (*) Sequence positions according to reference strain NL-001.

Table 2.9 pTRiEX Vector sequencing primers

Primer	Sequence 5'-3'	Source
TRIEX UP	GTT ATT GTG CTG TCT CAT CA	NOVAGEN
TRIEX 2 R	AAT TAC CAC ACC AGC CAC CAC CT	Dr P.Heinen HPA
POLH105	ATA GTT GCT GAT ATC ATG GAG	Dr P.Heinen HPA
FC-60R	TTC AGG TGC TGG GCA CGG TGG	This study

Table 2.10 hMPV PCR 2 Primers

Primer	Gene	Position*	Sense	Sequence 5'-3'	Source
HMPVF283F	F	3334-3353	+	ATTGAAAATCCCAGA CAATC	This study
HMPVF575R	F	3645-3626	-	TCTGTTGAATTGACTG AAGC	This study
HMPVF3052F	F	3052-3071	+	ATGTCTTGGAAAGTG GTGAT	This study

Footnotes: (*) Sequence positions according to reference strain NL-001.

Chapter 3

Development and application of detective methodology for hMPV

3.1. Introduction

The use of RT-PCR for the diagnosis of respiratory viruses is fast becoming the gold standard in diagnostic laboratories as it offers a number of advantages over conventional methods such as virus isolation in cell culture, and immunofluorescence (Dumler & Valsamakis, 1999, Tang, 2003, Tang et al., 1997).

Cell culture isolation is time consuming and is dependent on obtaining good quality samples with viable virus. This technique is also limited by the use of cell lines which, often, do not support the growth of many viruses. Growth of hMPV in tissue culture has proved to be difficult, with very few reports of successful laboratory isolation (Peret et al., 2002, van den Hoogen et al., 2001). RMK and LLC-MK2 cells have been reported to give the best results, but requires incubation periods of up to 3 weeks often without cytopathic effect (CPE). The use of primary cell lines such as RMK which often provide the best opportunity for isolating a broad spectrum of viruses, however, is being phased out in part, due to ethical issues surrounding the use of such cell lines, but also the advancement in other technologies and the need for more rapid tests.

Immunofluorescence is also frequently used in diagnostic laboratories, and while it is a simple technique that provides a rapid result it is not as sensitive as molecular techniques, results may be subjective and high throughput screening is labour intensive (Bellau-Pujol et al., 2005). RT-PCR provides rapid clear cut results for high throughput screening and offers increased sensitivity and specificity over conventional assays . Taking these factors into account it is clear that molecular methods such as RT-PCR provides the best opportunity for the detection of hMPV.

At the time of starting this thesis only two RT-PCR methods had been reported for the detection of hMPV. These targeted the L polymerase gene and Fusion gene (Jartti et al., 2002, Peret et al., 2002, van den Hoogen et al., 2001). At the Health Protection Agency, Colindale, an RT-PCR targeting the F gene had also been developed and validated for the detection of hMPV and was used to generate much of the data presented in this chapter (hMPV PCR1)(Nicholson et al., 2006). However, the increasing availability of sequence data means that such assays require constant evaluation and refinement to ensure they offer

the best opportunity for detecting all possible strains of the virus. Therefore, as reported in this chapter a second RT-PCR was developed, based on the more up to date sequence data available at the time (hMPV PCR 2).

Being a newly identified human pathogen a considerable amount of data regarding hMPV epidemiology has been generated since its discovery. However at the start of this PhD knowledge of hMPV epidemiology was very limited. As described in chapter 1 hMPV had been discovered in children with RTI that were negative for other respiratory pathogens (van den Hoogen et al., 2001). An Australian study had also detected hMPV in 2 out of 200 (1%) samples obtained from children with an unknown cause of viral pneumonia or bronchiolitis (Nissen et al., 2002) and in 40 out of 535 (7.4%) samples obtained from people in the general community, 38 of which were children (Howe, 2002). In the UK 9 out of 408 (2.2%) samples from patients in the community with influenza like illness (ILI), negative for other viral pathogens were positive for hMPV, 8 of which were adults between the ages of 20 to 75 years (Stockton et al., 2002).

Given the limited knowledge of hMPV it was vital that the appropriate tools where developed and applied to the relevant clinical samples to assess the prevalence of hMPV in the UK population. This data will help to determine the contribution hMPV makes to respiratory illness in the UK and identify the most at risk groups of infection. It is also vital for assessing the potential value of vaccine and or antiviral development.

At the health protection Agency, Colindale the Respiratory Virus Unit has a number of studies in place for the surveillance of respiratory viral infections in different populations within the UK.

The surveillance of both upper (tonsillitis, sore throat, otitis media, common cold, sinitus) and lower (ILI, Pneumonia, bronchitis, laryngitis) respiratory tract infections is coordinated by the Royal College of General Practitioners (RCGP) (Fleming et al., 2005) . ILI is of a particular interest because of the severity of the disease. ILI is an acute lower respiratory tract infection described as an acute illness with high fever, dry cough and muscle pains with a duration of ≤ 5 days appearing at the same time as several other cases. The seasonal trend of ILI is highly consistent with the detection of influenza which contributes enormously to respiratory illness over the winter months(Fleming et al., 2005).

As such the Health Protection Agency, Colindale collaborates with the RCGP to monitor the incidence of influenza associated with ILI in the community (Ellis et al., 1997). The incidence of RSV in this population has also been successfully monitored and has shown to play a more important role in the incidence of ILI than previously thought (Stockton et al., 1998), however more than 50% of these samples test negative for both influenza and RSV each year (Zambon et al., 2001).

Surveillance of influenza and RSV in hospitalized children is also carried out in collaboration with The Leicester Royal Infirmary Children's Hospital; the only facility within the county providing inpatient pediatric care to a total population of about 1 million inhabitants of all ages, including 68,000 children ≤71 months of age (Nicholson et al., 2003).

The investigation of the prevalence of hMPV in the general population and hospitalised children, as well as others should provide a good overall picture of the extent of hMPV prevalence in people with RTI in the UK. Reported here is the development of a RT-PCR for the detection of hMPV in clinical samples and its application to an epidemiological study of hMPV in the UK.

3.2. Aims

The aim of the work reported in this section of the thesis was to design, optimise and validate a PCR specific for the detection of all four known subtypes of hMPV, able to detect virus from clinical material such as nose and throat swabs.

The second aim of this section of work is to determine the extent to which hMPV contributes to respiratory disease in the general community, hospitalised, elderly and immunocompromised populations throughout the winter months, by applying the hMPV PCR to clinical samples from patients presenting with influenza like illness or acute respiratory tract infection.

3.3. Development of detection methodology for hMPV

3.3.1. Virus growth

In order to provide positive control material for the assay it was necessary to culture the virus in vitro. Due to the fastidious nature of this virus a number of different cell lines under a number of different conditions were tested for their ability to support the growth of hMPV from clinical samples. Cell lysate material was tested using hMPV PCR 1, to determine whether hMPV growth was supported.

3.3.1.1. Culture conditions Tested

- Cell lines tested: LLC-MK2, HEP2, HEP2V, A549, Vero, BSC-1, RMK
- Trypsin concentration: 0 – 2μg/ml
- Temperatures tested: 33-37^{0}C
- Incubation Period: 3-21 days
- Cell confluency at inoculation: 60-100%
- Culture vessels: Flask or tubes – Stationary or rolling.

3.3.1.2. Virus harvesting techniques tested

- Cell scraping with and without sonication.
- Mechanical smashing of cells using glass beads with and without sonication,
- Freeze thawing

3.3.1.3. Results of virus propagation

Over 50 different clinical samples known to be positive for hMPV by RT-PCR1 were inoculated on to the different cell lines, under the varying conditions listed above. Only three clinical samples yielded growth of hMPV in vitro that was detectable by RT-PCR.

Virus growth occurred only in LLC-MK2 cells incubated at 37^{0}C in the presence of 1.5μg/ml of trypsin. Propagation was limited to only 4 or 5 passages of the virus before it was no longer detectable by RT-PCR. Some CPE was observed with a couple of the isolates as illustrated in Figure 3.1, where cells became refractory and progressed to rounding up and detaching from the monolayer. However, generally no CPE was seen, and

viruses were blindly passed on to fresh cells. With the exception of cell line, and the presence of trypsin there appeared to be no advantage in particular to any of the above conditions tested, and therefore appeared to be more dependent on the quality of the clinical sample used. Immunofluorescence analysis of cell culture grown hMPV is shown in chapter 5 page197.

Uninfected LLC-MK2 Cells HMPV Infected LLC-MK2 Cells

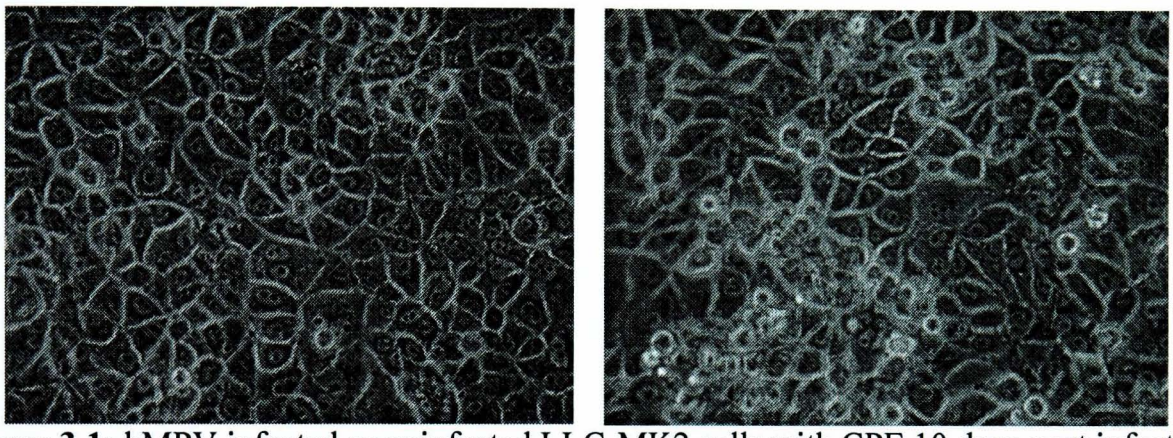


Figure 3.1: hMPV infected or uninfected LLC-MK2 cells with CPE 10 days post infection.

3.3.2. HMPV Positive Control Material

All hMPV positive control material used for molecular work, was generated in cell culture as described in Chapter 2 from a clinical isolate. The isolate came from a 4 month old male, hospitalised with bronchiolitis and upper respiratory tract infection. Sequence and phylogenetic analysis places this isolate as an A1 subtype (Chapter 4). The control material used was passage 3 cell lysate grown in LLC-MK2 cells. It was not possible to quantitate the amount of virus present as the poor growth properties of the virus did not allow for quantitative assays to be developed.

3.3.3. hMPV PCR 1

hMPV PCR 1 developed by Dr Joanne Stockton at the Heath Protection Agency Colindale was currently in use at the start of this PhD and has been used to generate much of the data presented in this chapter.

Nucleic acid extraction and cDNA synthesis was performed as described in Chapter 2. Details of this PCR are described in chapter 2 and can also be found in (Nicholson et al., 2006). Table 3.1 and Figure 3.3 shows the hMPV PCR primer sequences and positions.

Table 3.1 : hMPV PCR 1 primer sequences

Table 3.1 : hMPV PCR 1 primer sequences

Primer name	Sequence 5' to 3'	Sense	Position
HMPVMF1499	GCGGCAATTTTCAGACAACG	+	3663-3682
HMPVMF2175	ACATGCTGTTCGCCTTCAAC	-	4358-4339
HMPVMF1897	CAGCAGCAGGGATCAATGTT	+	4061-4080
HMPVMF2166	TCGCCTTCAACTTTGCTTAG	-	4349-4330

Footnotes: Primer positions are based on reference strain NL00-1 accession number AF371337

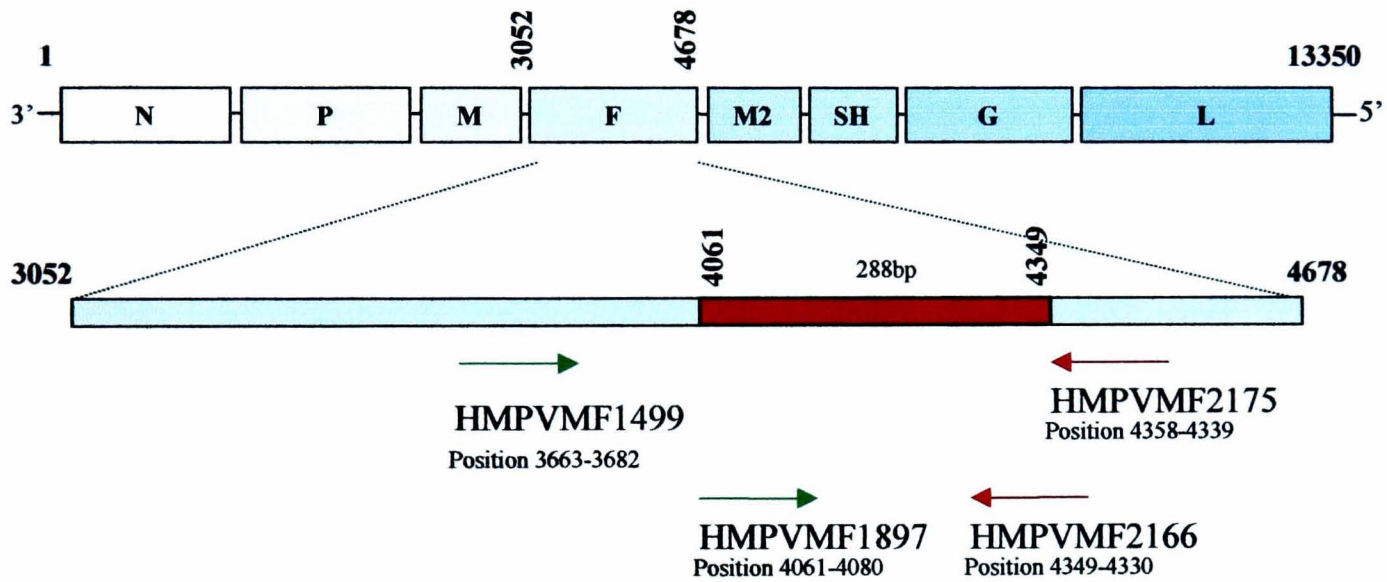


Figure 3.2: Schematic representation of PCR 1 target region and primer positions. Primer positions are based on reference strain NL00-1 accession number AF371337

3.3.4. Development of hMPV PCR 2

Before the identification of SARS coronavirus as the aetiological agent of the SARS outbreak in 2003 (Fouchier et al., 2003), a number of other respiratory viruses were identified as possible causative agents, including hMPV (Chan et al., 2003, Chan et al., 2004). As a result, more hMPV sequence rapidly became available which allowed the design of improved assays

The F gene was selected for detection of hMPV due to the availability of sequence. Primers were selected based on sequence analysis, the ability to create primer pairs for both single round and nested PCR approaches, amplicon length melting temperature, GC content,

primer combinations were tested against laboratory grown material. Nucleic acid extraction and cDNA synthesis was performed as described in Chapter 2.

Using standard PCR buffer conditions. For the first round 20 µl of hMPV cDNA was added to 80 µl of PCR mix (20mM Tris-HCL (pH 8.0), 50mM KCL, 1mM $MgCL_2$). For the second round PCR, 2 µl of the primary round was added to 48 µl of PCR mix with the addition of 0.2mM of each dNTP. An annealing temperature of 50^0C was used. Both primary and secondary round products were visualised by agarose gel electrophoresis. All primer combinations tested yielded products of the expected size and conformation of the correct product was achieved by sequence analysis of the amplicon. The primer set chosen however had the greatest specificity in both the primary and secondary round of the PCR.

Optimisation of the PCR buffer and cycling conditions was performed for each round of the PCR. The buffer components optimised included $MgCl_2$, KCL and Tris-HCL concentration and pH and was performed using the Stratagene PCR Optimisation Kit. For both the primary and secondary round the final buffer conditions where optimised to 10mM Tris HCL, pH9.2, 1.5mM $MgCl_2$, and 75mM KCL. Optimisation of the annealing temperature was performed using a PTC 225 gradient thermocycler varying the annealing temperature over 12 increments from 45^0C to 60^0C .

For the primary round PCR primer set the specificity and sensitivity of the PCR increased with annealing temperature. The optimal temperature was set at 58°C. At lower annealing temperatures extra bands and smearing were visible on the gel as decreased stringency allowed miss-priming elsewhere in the genome. For the second round primer set the optimal temperature was 51°C which give slightly greater sensitivity over the other temperatures tested.

3.3.5. Summary of hMPV PCR 2 optimised conditions

3.3.5. Summary of hMPV PCR 2 optimised conditions

Table 3.2 Summary of hMPV PCR 2 optimised conditions.

Variable	Optimised condition
First round PCR primer set	HMPVF3052F & HMPVF575R
Second round PCR primer set	HMPVF283F & HMPVF575R
PCR buffer conditions	10mM Tris HCL
	1.5mM $MgCl_2$
	75mM KCL
	pH9.2
Primary round PCR Annealing temperature	58°C
Secondary round PCR annealing temperature	51°C

Footnotes: For full description of protocol see chapter 2, Materials and methods.

Table 3.3 hMPV PCR2 primer sequences and positions.

Primer name	Sequence 5' to 3'	Sense	Position
HMPVF3052F	ATGTCTTGGAAAGTGGTGAT	+	3052-3071
HMPVF283F	ATTGAAAATCCCAGACAATC	+	3334-3353
HMPVF575R	TCTGTTGAATTGACTGAAGC	-	3645-3626

Footnotes: Primer positions are relative to reference strain NL00-1 accession number AF371337

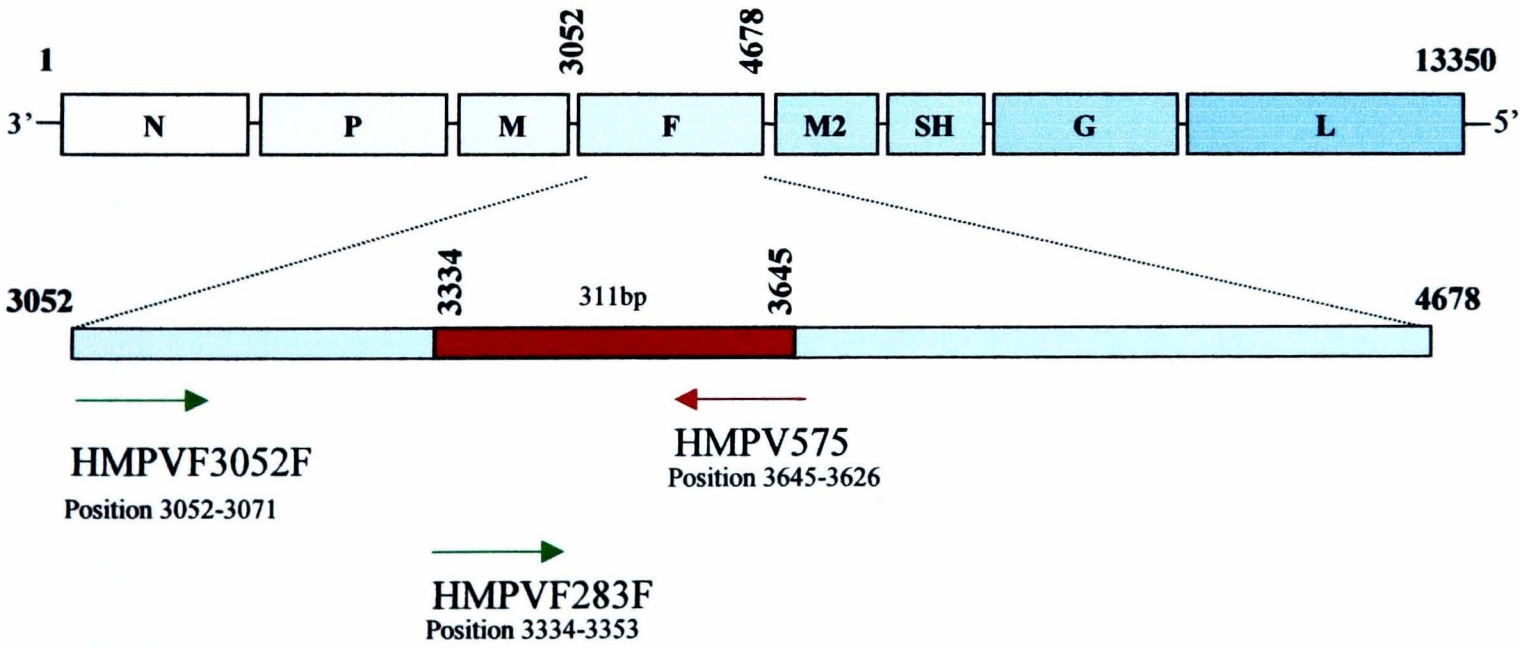


Figure 3.3: Schematic representation of PCR 2 target region and primer positions. Positions based on reference strain NL00-1 accession number AF371337.

To assess the specificity of hMPV PCR 2 cDNA generated from a number of other common respiratory viruses including; influenza H1; influenza H3; influenza B; and RSV A and B was tested. Nose and throat swabs samples, known to be either positive or negative for influenza RSV or hMPV where also tested (Figure 3.4). A small amount of low molecular weight smearing was observed in clinical samples however this did not result in the production of defined bands and did not interfere with interpreting the results.

To evaluate the specificity of the PCR further products amplified from a number of different clinical samples where sequenced, all of which yielded hMPV sequence.

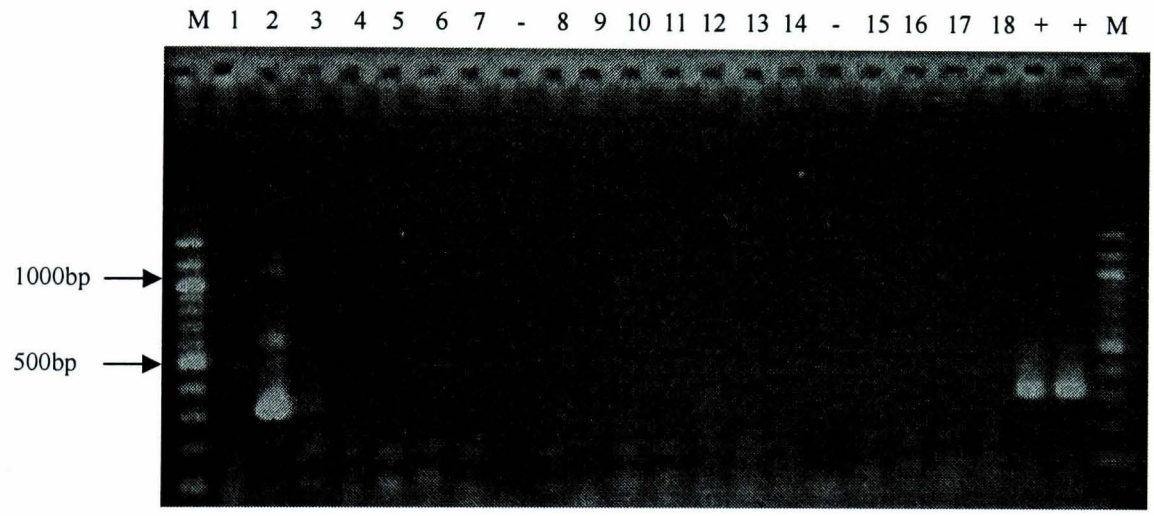


Figure 3.4: A typical example of the hMPV PCR on clinical material seen in this study. (M) 100bp molecular weight marker numbered samples, combined nose and throat swab material from patients presenting to GP with ILI and submitted to the HPA for influenza surveillance. (-) Negative water controls (+) Positive control. Lane 2 shows a typical positive reading.

3.3.7. hMPV PCR 2 Sensitivity

The absolute sensitivity of hMPV RT-PCR 2 in relation to the number of PFU of virus could not be determined due to difficulties in growing the virus in tissue culture. RNA transcripts of the F gene were therefore synthesised in vitro using the PTM1 vector into which the relevant portion of the hMPV F gene was cloned (Provided by Laura Beasley and Dr Bermingham HPA Colindale generated from an A1 subtype virus obtained from Dublin; Ireland). The vector was linearised using the Sma1 restriction site located downstream of the hMPV F gene. Transcripts were synthesised in vitro by T7 RNA polymerase under the control of the T7 promoter located upstream of the cloned hMPV F gene fragment. The plasmid DNA was removed by digestion with recombinant RNAse

free DNAse, and passed through a Qiagen RNAEasy clean up column. The quantity of RNA was determined by spectrophotometry and the number of copies of the transcripts per µl determined using the calculations given in chapter 2 (2.3.18). A 10 fold dilution series of the RNA transcripts was made, and used to generate cDNA, and tested by hMPV PCR 2 (Figure 3.5).

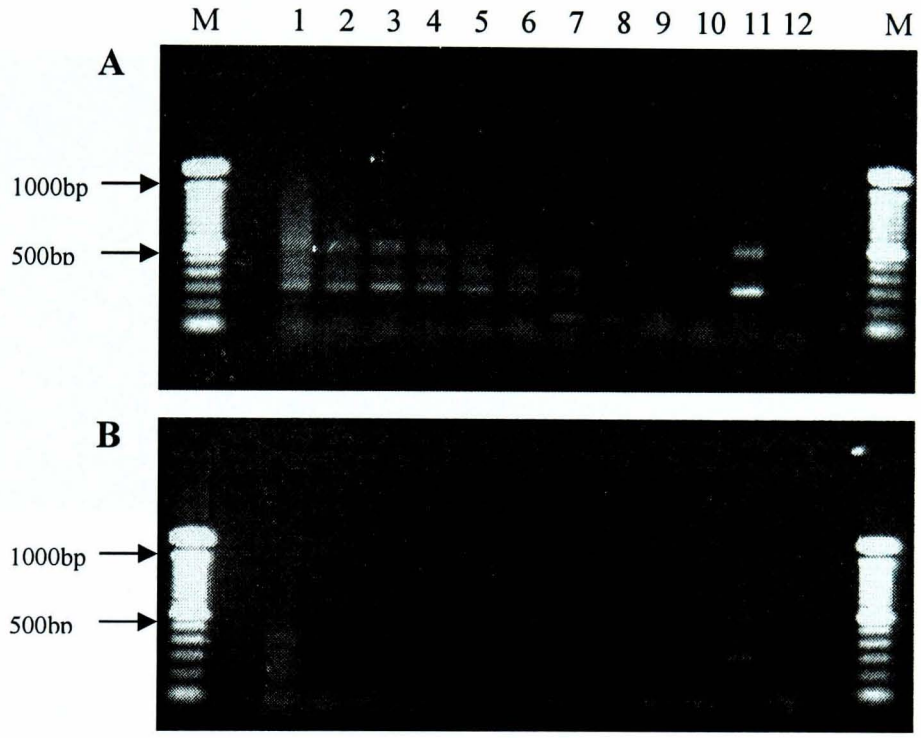


Figure 3.5: Analysis of hMPV PCR 2 sensitivity using in vitro synthesised RNA transcripts. M is a molecular weight marker. (A) Lanes 1 to 9 are a 10 fold dilution series of the synthetic transcripts starting at 1:10 which contained $1x10^{10}$copies of RNA. (B) Control of template digestion. A 10 fold dilution series starting 1:10 of material which has undergone the whole transcription process, without the addition of reverse transcriptase to ensure that the DNAse digestion was complete and amplicons were not being generated as a result of DNA vector carry through. In both A and B lanes 10 and 12 are negative controls and lane 11 is a positive PCR control.

Results show that that the hMPV PCR 2 has a limit of detection of $1x\ 10^4$ copies/ reaction of the RNA transcripts which equates to roughly $1.8x10^6$ copies/ml. Whilst in figure 10B no amplicons are generated with the exception of a slight smear in lane 1 indicating that there is no residual DNA vector left following DNAse treatment. There were however 3 bands visible in the lanes containing transcripts the lowest of 311bp being the expected amplicon size for the second round PCR. The highest MW band of approximately 700bp is also visible in the PCR positive control and is the correct size for the primary round product. The third fainter band of approximately 400bp is most likely to be a result of transcript degradation.

The plasmid itself was also applied to the PCR in a 10 fold dilution series, and the number of copies determined. Figure 3.6 shows that the hMPV PCR 2 was able to detect up to $1x10^4$ copies of the vector which equates roughly to $1x10^6$ copies /ml.

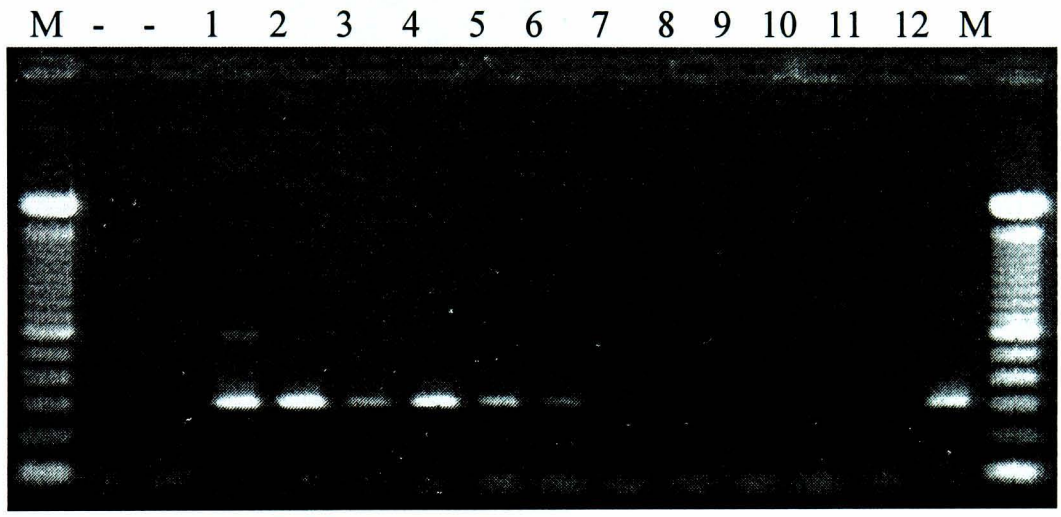


Figure 3.6: Analysis of the absolute sensitivity of the by hMPV PCR2. M is a molecular weight marker, lanes 1 through to 10 are a 10 fold dilution series of the vector starting from $1x10^{10}$ copies through to $1x10^1$ copies per reaction. lane 11 is a negative control and lane 12 hMPV positive control.

3.3.8. hMPV PCR 1 Versus hMPV PCR 2

hMPV PCR 2 was compared with hMPV PCR 1 using cDNA from laboratory grown material to determine the relative sensitivities of the two assays. Figure 3.7 clearly shows that hMPV PCR 2 out performs hMPV PCR 1 in terms of sensitivity and specificity. For

PCR 1 there are a number of bands other than that of the expected size of 300bp present in lanes 5 through to 8.

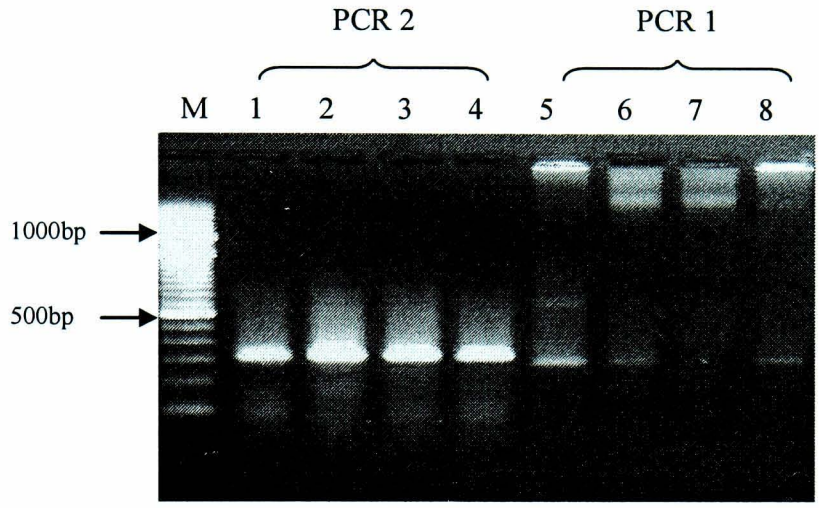


Figure 3.7: hMPV PCR1 Vs PCR 2. second round PCR products generated from a dilution series (Neat, 1:10, 1:20 and 1:40) of hMPV cDNA was analysed by DNA gel electrophoreses. Lanes 1 to 4 contain hMPV PCR 2 products. Lanes to 8 contain hMPV PCR 1 products. (M) 100bp molecular weight marker. (1) hMPV PCR 2 Neat cDNA. (2) hMPV PCR 2 1:10 cDNA. (3) hMPV PCR 2 1:20 cDNA. (4) hMPV PCR 2 (1:40) cDNA. (5) hMPV PCR 1 Neat cDNA. (6) hMPV PCR 1 1:10 cDNA, (7) hMPV PCR 1 1:20 cDNA, (8) hMPV PCR 1 1:40 cDNA.

3.4. Clinical Studies

To address the question of the incidence of hMPV, and its contribution towards burden of respiratory illness in the community and other populations, clinical respiratory samples, mostly combined nose and throat swabs or nasopharyngeal aspirates (NPA), from defined clinical studies, were tested for hMPV, and other respiratory viruses by PCR. An outline of each of the clinical studies, target population, clinical setting, sample type and number is shown in Table 3.4.

The samples were received and processed as described in chapter 2. Where influenza and RSV was tested as part of the national surveillance programme the remaining cDNA was stored at -20^0C until testing for hMPV. When no cDNA was available nucleic acid was re-extracted from the original sample.

For hMPV testing either hMPV PCR 1 or hMPV PCR 2 were used. hRSV and Influenza were detected using the multiplex assay described elsewhere (Stockton et al., 1998). Coronavirus, Rhinovirus and other Enteroviruses were detected in the Leicester Royal Infirmary study using assays developed at the HPA Colindale.

Testing of samples for influenza and RSV was carried out as part of routine influenza surveillance by the Respiratory Virus Unit, HPA Colindale, who kindly provided the data for analysis. Coronavirus, Rhinovirus and Enterovirus data was kindly provided for analysis by Dr Hosler and Dr Lock of the Respiratory Virus Unit, HPA Colindale. All data analysis presented in this thesis was performed by myself.

Table 3.4: Summary of studies used to assess the prevalence of hMPV in clinical cohorts in the UK.

Study	Study Cohort	Age	Winter Season	Study Description	Sample Type	Sample Number	HMPV PCR used
RCGP	Community Acquired All Ages with ILI	All Ages	2001-2002	Royal College of General Practitioners Influenza surveillance scheme. General practitioners from England enrolled in the scheme take samples from patients from the general community presenting to them with influenza or influenza like illness. For the investigation of influenza strain surveillance (Stockton et al., 1998, Zambon et al., 2001)	NT Swab	389	1
			2002 - 2003			372	2
			2003 - 2004			655	2
Leicester Royal Infirmary	Hospitalised Children with ARTI	≤ 5 Years	2001-2002	Children under the age of 5 years admitted to the Leicester Royal Infirmary in the UK with acute respiratory tract infections.(Nicholson et al., 2003)	NPA	510	1
			2002 - 2003				2
RCGP/ Aventis	Community Acquired Elderly with ARTI	≥ 65 Years	2001-2002	Burden of disease caused by RSV in community dwelling elderly > 65 years old.	NT Swab	264	1
Royal Free	Hospitalised Immuno compromised with ARTI	All Ages	2002-2004	Surveillance of acute respiratory illness in patients in the immunocompromised ward of the Royal Free Hospital.	NPA	134	2

The RCGP ILI index is the number of people per 100,000 of the UK population presenting to General practitioners with influenza or influenza like illness (Table 3.4) and is a useful guide to the severity/ timing of ILI for the given year, and as such, has been included in the following analysis.

Table 3.4: RCGP ILI Index and Influenza Activity.

RCGP Index	Category
< 50	Baseline Influenza Activity
50 - 200	Normal Influenza Activity
>200	Epidemic Influenza Activity

3.5. Community Acquired Infections

1416 clinical samples (combined nose and throat swabs) submitted as part of the national surveillance program for ILI were tested prospectively for influenza and RSV, and retrospectively for hMPV. Samples were taken between October and March for 3 successive seasons, 2001-2002 (Year 1), 2002-2003 (Year 2) and 2003-2004 (Year 3). Year 1 was analysed for hMPV using hMPV PCR 1 and year 2 and 3 using hMPV PCR 2.

The total number of incidences of hMPV, RSV and Influenza over the 3 years is summarised in table (Table 3.5). In total only 43% of the samples were positive for either hMPV, RSV or influenza leaving the remaining 57% without identification of a possible aetiological agent. Overall, hMPV accounted for 4.8 % of the total number of samples (11.2 % of those with a virus detected). The incidence of RSV is more than twice that of hMPV, and influenza almost 12 times that hMPV.

The percentage of hMPV infections increased from Y1 to Y2 from 3.4% to 5.1%, which, in part, may be due to the introduction of hMPV PCR 2 in 2003. The percentage of hMPV was similar for Y2 and Y3. Influenza activity was similar for Y1 and Y2, however, increased by more than three times in Y3 accounting for almost 38% of infections and is responsible for the increased samples number received that year. Incidences of RSV, increased dramatically in Y2 to over 25%, and then fell again in Y3 to levels lower than that of hMPV.

Table 3.5: Community Acquired infections 2001:2004: Percentage of hMPV, RSV and Influenza cases detected. 95% confidence intervals calculated using STATA Version 8.2 (STATACorp, USA).

Year	Total No of Samples	hMPV %	RSV %	Influenza %	Unknown %	Dual Infections %
01/02 (Y1)	389	3.5	6.2	19.2	70.9	0
02/03 (Y2)	372	5.1	25.3	20.4	49.2	2.9
03/04 (Y3)	655	5.3	4.1	37.7	52.8	1.5
Total	1416	4.8	10.2	28.1	28.1	1.5
95% CI		3.7-6.0	8.7-11.9	25.7-30.5	54.2-59.4	0.9-2.3

A total of 21 dual infections were identified over the three years and analysis of these is shown in Table 3.7. Eighteen were dual infections of hMPV with either RSV or Influenza. This accounts for 26.4 % the total number of hMPV positive samples.

Table 3.6: Community Acquired Dual Infections.

Dual Infection	Number of Samples (% of Total)
HMPV and RSV	13 (0.9%)
HMPV and Influenza	5 (0.3%)
RSV and Influenza	3 (0.2%)

3.5.1. Weekly Distribution

The week by week analysis of the distribution of hMPV compared with RSV and influenza for the 3 seasons is shown in Figure 3.9. The distribution of the samples received throughout the 3 seasons is reflected in the RCGP index, and is similar in both Y1 and 2 of the study with a peak in clinical activity between weeks 1-10. In Y3 , however the vast majority of illnesses occurred in the early part of the season between weeks 43-1 due to the earlier circulation of influenza that year.

hMPV appeared at a fairly consistent rate throughout all 3 seasons, peaking slightly in week 50, weeks 1 and 3, and week 49 in Y1, 2 and 3 respectively. The length of time that hMPV occurred through out the season varied from 15 to 24 weeks over the 3 years. hMPV activity appeared therefore to be unaffected by the shift in ILI in Y3.

The RSV season started 8-10 weeks earlier than the influenza season in Y1 and 2 peaking in weeks 1 and 2 respectively. However, in Y3 the influenza season preceded that of RSV by 7 weeks, reflecting the shift in ILI . The RSV season was not affected by this shift and peaked in week 2, which is consistent with the previous 2 seasons. The influenza seasons mirrored the RCGP ILI index over all 3 seasons peaking in week 3, 8 and 46 in Y1, 2 and 3 respectively.

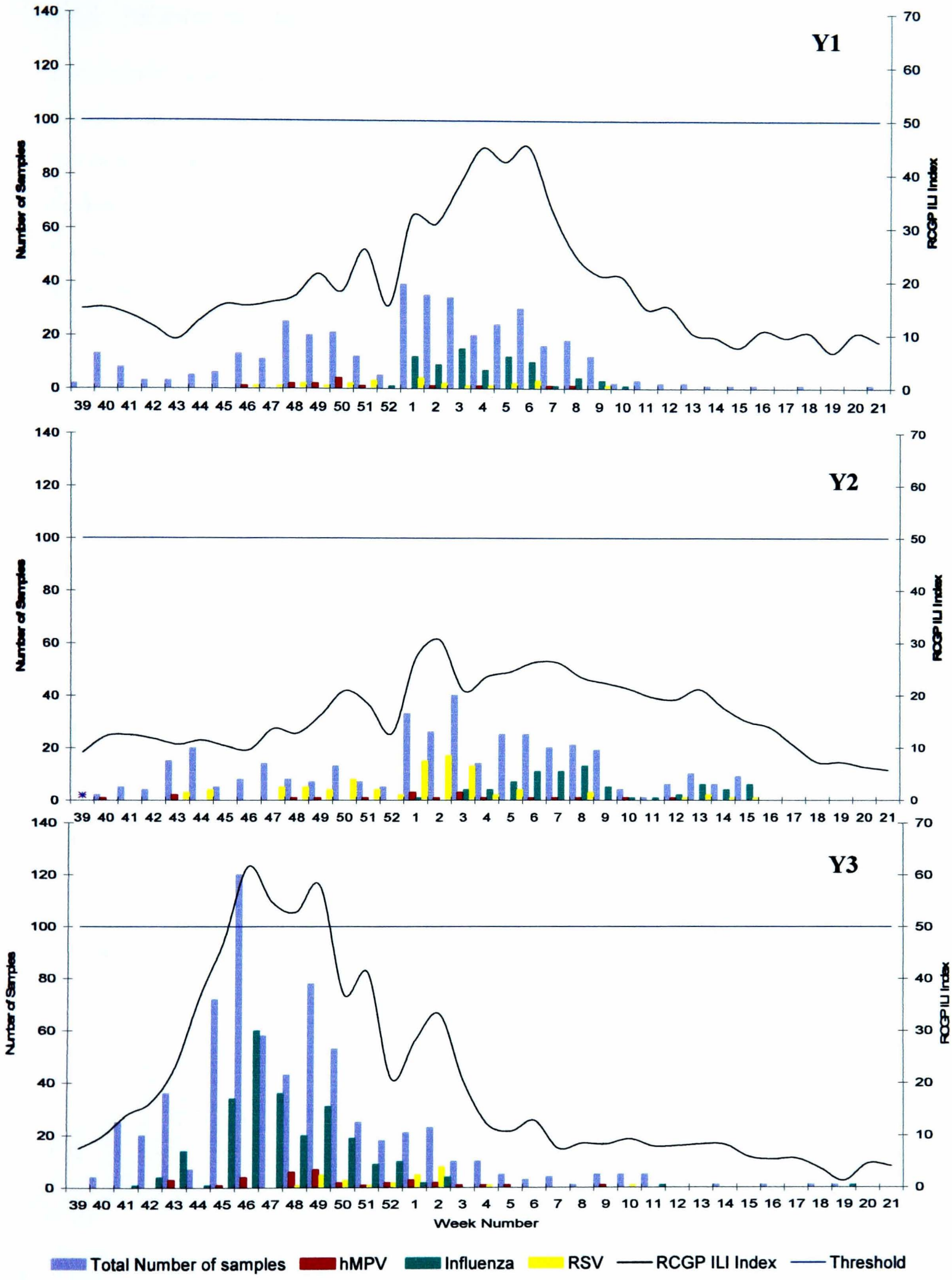


Figure 3.8: Weekly distribution of community acquired hMPV, RSV and influenza infections over 3 winter seasons. 2001-2002 (Y1), 2002-2003 (Y2), and 2003-2004 (Y3).

3.5.2. Monthly Distribution

The monthly distribution of hMPV, RSV and influenza over the thee seasons is shown in Figure 3.10. The largest number of samples were received in January for Y1 and 2, and November for Y3 reflecting the trend of the RCGP ILI Index. hMPV was detected throughout all three 3 winter seasons with a peak incidence in December for winter seasons Y1 and Y3, and January in Y2. RSV peaked in January in all 3 years, while influenza peaked in January, February and November in Y1, 2, and 3 respectively.

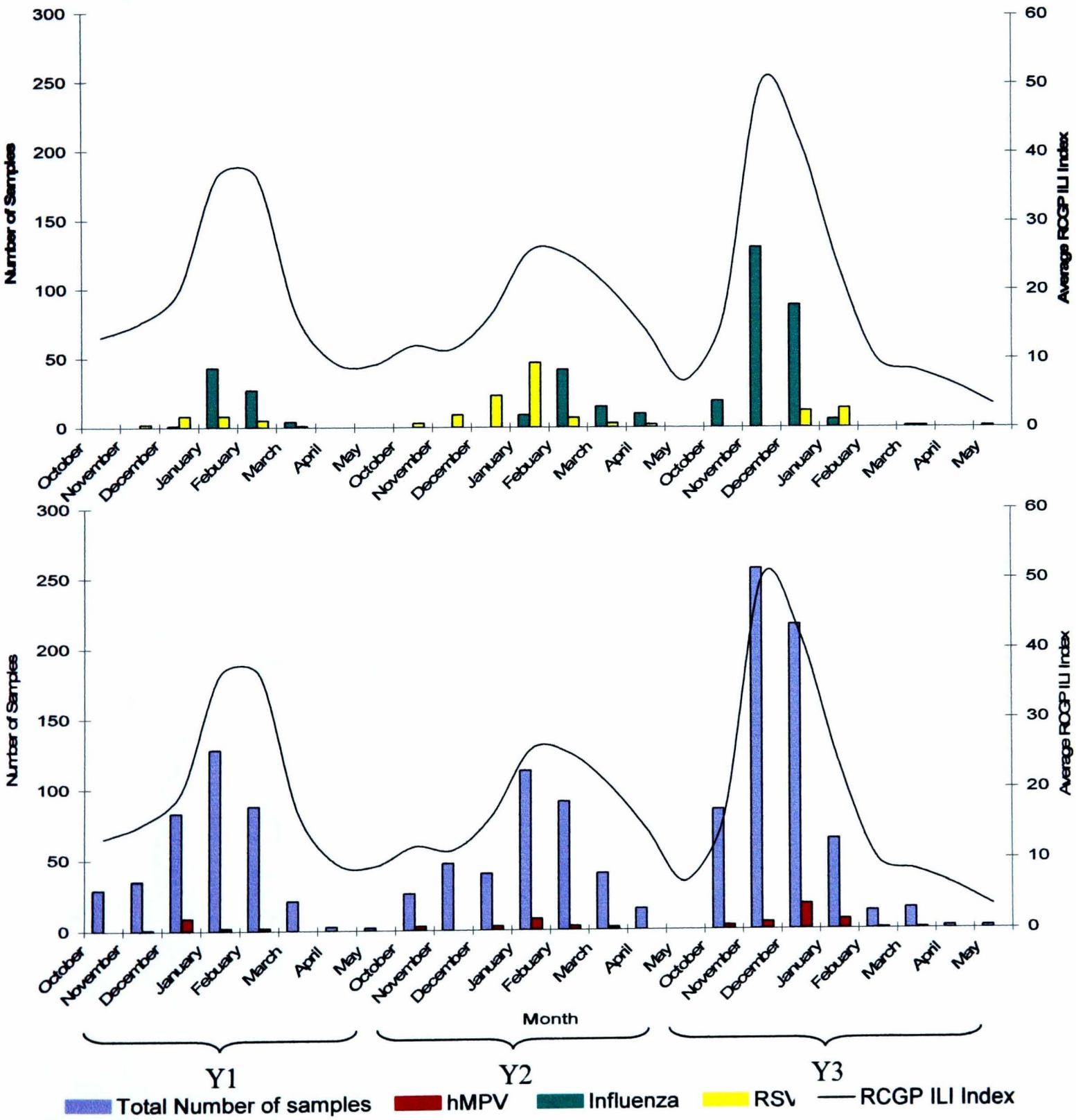


Figure 3.9: Monthly distribution of community acquired hMPV influenza and hRSV Infections over 3 consecutive winter seasons. 2001-2002 (Y1), 2002-2003 (Y2), and 2003-2004 (Y3).

3.5.3. Age Distribution

The proportion of patients with hMPV, RSV or influenza infections for each age group of the community over the 3 winter seasons is shown in Figure 3.11, and the number hMPV, RSV and Influenza infections for each season shown in Figure 3.11. Over the 3 seasons the age of the patients ranged from 2 days to 99.2 years old, with an average age of 32.8 years reflecting the age distribution of the population sampled. All 3 viruses were detected in all age groups. Proportionally, hMPV was most prevalent in the 1-4 year age group accounting for 16.2% of all hMPV infections. hMPV occurred in roughly equal proportions in all age groups with the exception of infants <1 year old, where no hMPV cases were detected in Y1 or 2 of the study.

As expected the largest proportion of RSV infections occurred in infants < 1 year old, and decreased in older children and adults, however it increased again slightly with age in adults 35 years and up. Y2 of the study was the exception, where a majority of RSV infections occurred in adults between the ages of 15-44.

Influenza infections also followed the expected trend for all 3 seasons occurring most frequently in school age children of 5-14 years old and decreased with age. Few influenza infections were detected in young children.

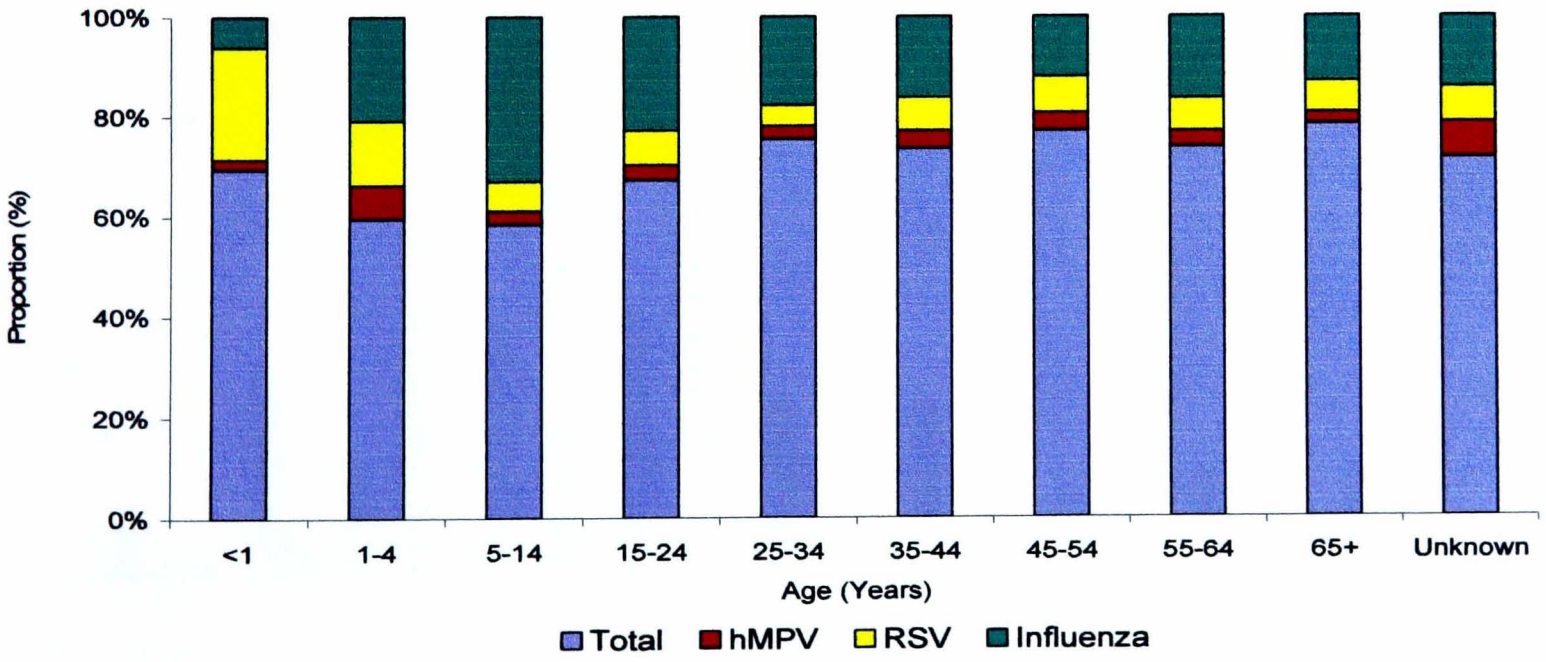


Figure 3.10: Proportional age distribution of hMPV, hRSV, and influenza community acquired infections taken over 3 consecutive winter seasons (2001-2004).

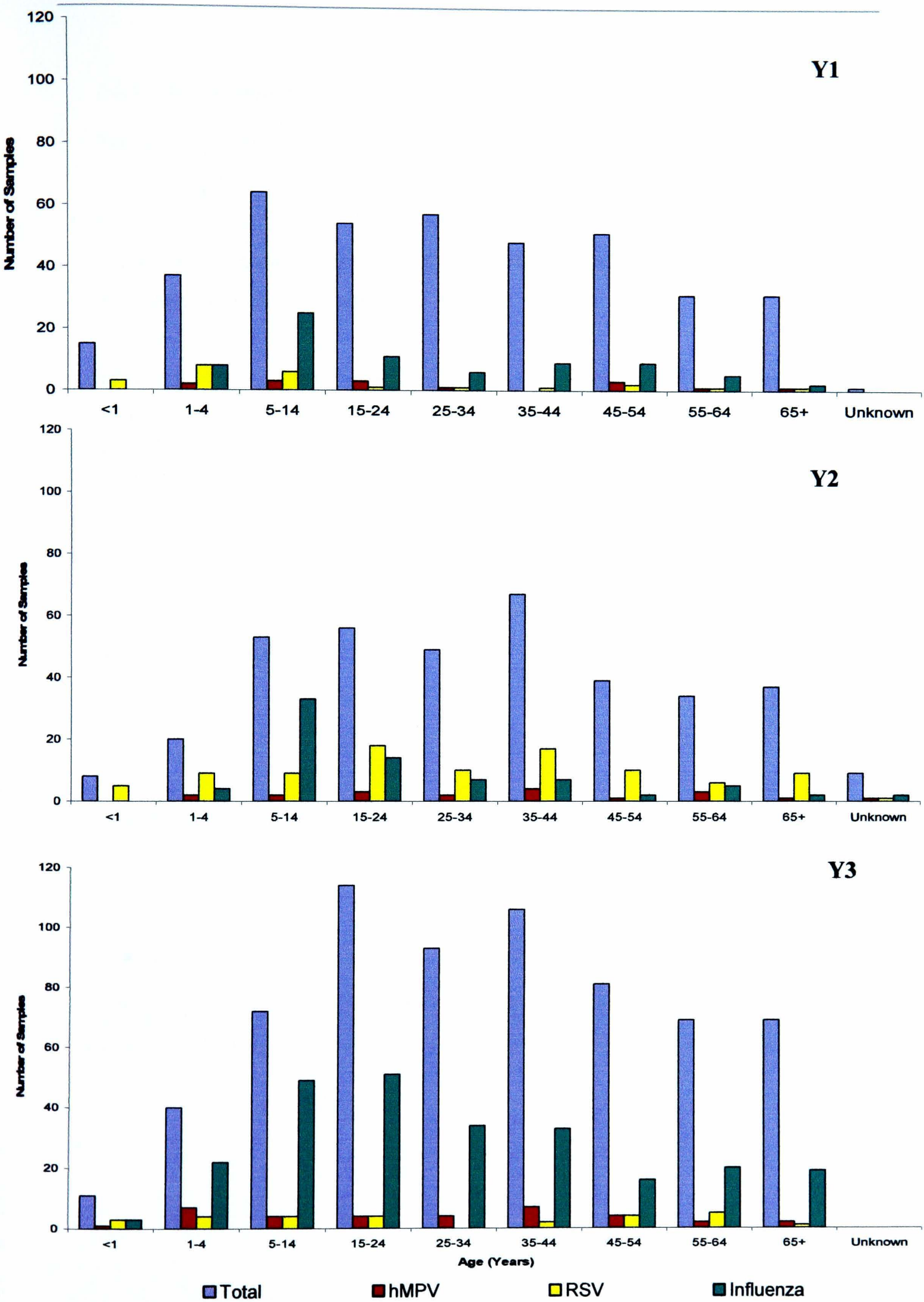


Figure 3.11: Age distribution of total number of samples recieved, hMPV, hRSV, and influenza community acquired infections over 3 consecutive winter seasons. 2001-2002 (Y1), 2002-2003 (Y2), and 2003-2004 (Y3).

3.5.4. Sex Distribution

The sex distribution of hMPV, RSV and influenza infections in the community over the 3 winter seasons is shown in Table 3.7. Overall just over 53% of the total number of samples were from female patients. The distribution of both hMPV and influenza infections is split at 50% between males and females. The majority (53.8%) of RSV infections, however, were detected in male patients.

Table 3.7: Sex distribution of patients with hMPV, h RSV and influenza community acquired infections taken over 3 consecutive winter seasons (2001-2004).

Sex	hMPV	RSV	Influenza	Total
Number of Males	33	78	192	**659**
% Grand Total	2.3	5.5	13.6	46.5
% of Males only	5.0	11.8	29.1	100
% Total virus	48.5	53.8	48.2	-
Number of Females	34	67	205	**753**
% Grand Total	2.4	4.7	14.5	53.8
% of Females only	4.5	8.9	27.2	100
% Total virus	50	46.2	51.5	-
Number Sex Not Known (SNK)	1	0	1	**4**
% Grand Total	0.07	0	0.07	0.3
% SNK only	25	0	25	100
% Total Virus	1.5	0	0.3	-
Total virus	**68**	**145**	**398**	
Grand Total				**1416**

3.6. Hospitalised Children

18 month study (2001/2003)

A total of 1171 clinical samples (nasopharyngeal aspirates) were taken over an 18 month period from October 2001 to March 2003, to investigate the cause of respiratory illness in children,< 6 years old, hospitalised with ARTI. The samples were tested prospectively for influenza and RSV, and retrospectively for hMPV. Y1 (October 2001-July 2002) was analysed for hMPV using hMPV PCR 1 and Y2 (August 2002- March 20003) using hMPV PCR 2. Other respiratory viruses mentioned were detected using PCR assays developed at the HPA Colindale.

The incidence of hMPV, RSV, influenza, coronavirus (CoV), rhinovirus (RV) and other enteroviruses (other than rhinovirus) (EV) is shown in Table 3.8. In total 57% of the samples had either 1 or more viruses detected leaving 43% without identification of a possible aetiological agent. Overall hMPV accounted for 5.7 % of the cases (9% of those with a virus detected). The percentage of hMPV infections increased from Y1 to Y2 from 4.4% to 7.1% respectively, which, in part, may be due to the introduction of hMPV PCR 2 in 2003. The incidence of RSV and RV infections were almost 4 times that of hMPV. As expected RSV was the predominant cause of infection in these children. Influenza was slightly higher that of hMPV and Other EV double, and again both viruses increase from Y1 to Y2. CoV infections accounted for half the number of hMPV infections and, unlike the other viruses, the number of CoV cases decreased from Y1 to Y2 by almost 50%.

Table 3.8: Hospitalised children; 18 month Study: Percentage of hMPV, hRSV and influenza cases detected. 95% confidence intervals calculated using STATA Version 8.2 (STATACorp, USA). (CV) Coronavirus, (RV) Rhinovirus and (EV) Other Enteroviruses except Rhinoviruses.

	Total	hMPV %	RSV %	Influenza %	CoV %	RV %	EV %	Dual %
01/02 (Y1)	613	4.4	16.8	6.8	2.9	14.8	11.3	5.4
02/03 (Y2)	558	7.2	24.9	7.9	1.6	22.2	8.8	10.2
Total	1171	5.7	20.7	7.3	2.3	18.3	10.1	7.8
95% CI	-	4.4-7.2	18.3-23.1	5.7-8.7	1.4-3.1	11.3-25.3	8.3-11.8	6.1-9.2

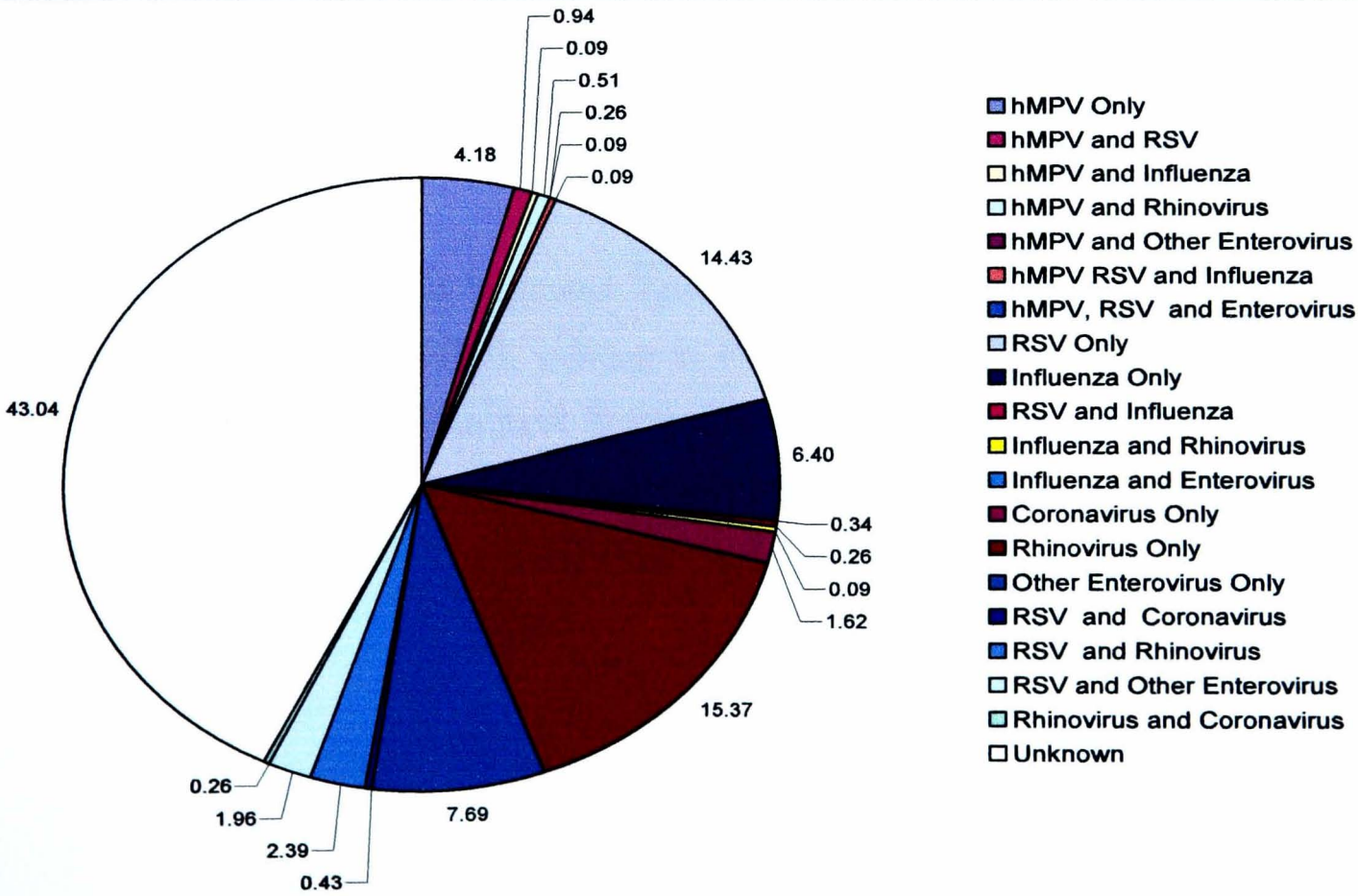
3.6.1. Dual Infections

3.6.1. Dual Infections

A total of 88 dual infections were identified and in 2 cases there where 3 viruses (Table 3.9). Of the hMPV positive samples 34% were dual infections, the largest number of which were with RSV. The greatest number of dual infections overall were with RSV and Rhinovirus. The contribution of each infection or dual infection to burden of illness is shown in Figure 3.12.

Table 3.9: Number of dual infections detected in children hospitalised with ARTI over the course of the 18 month study.

Dual Infection	Number of Samples
HMPV and RSV	11
HMPV and Influenza	1
hMPV and Rhinovirus	6
HMPV and other Enteroviruses	3
HMPV, RSV and Influenza	1
HMPV, RSV and other Enteroviruses	1
RSV and Influenza	4
RSV and Coronavirus	5
RSV and Rhinovirus	28
RSV and other Enteroviruses	23
Influenza and Rhinovirus	3
Influenza and other Enteroviruses	1
Rhinovirus and Coronavirus	3



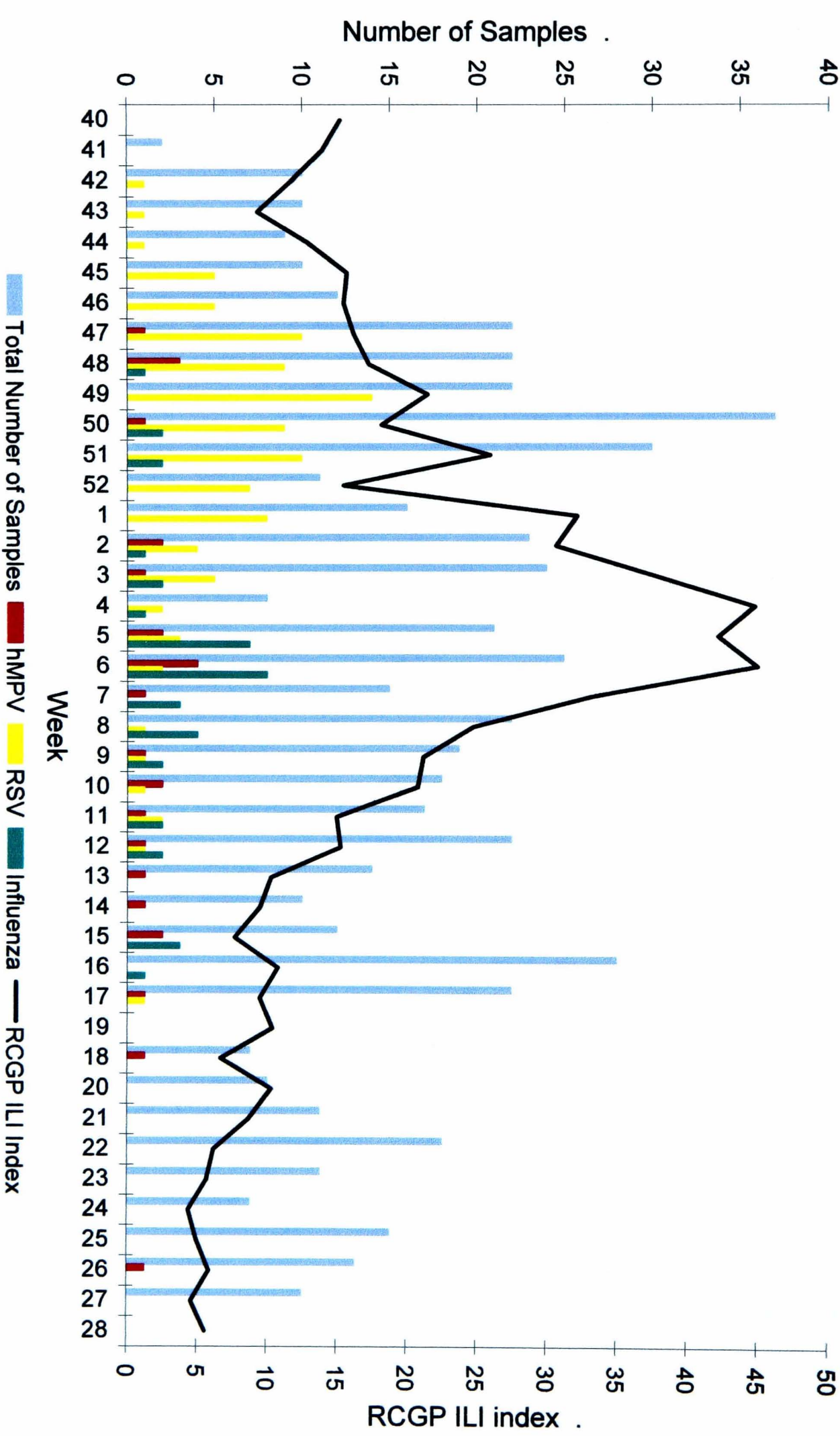
3.6.2. Seasonality

The monthly distribution of hMPV, RSV and Influenza is shown in Figure 3.14 and the weekly distribution in Figure 3.13. The period studied spans 18 months from October 2001 until march 2003, and includes 2 winter seasons and 1 summer. Samples were received for all months with the exception of August 2002 when respiratory virus activity is typically at its lowest. Based on the samples received the incidence of ARTI in children requiring hospitalisation reflects the RCGP ILI index for the community with the largest number of samples received in December / January for Y1 and 2 respectively.

hMPV was detected throughout the 18 months with the exception of October Y1 and July and August Y2. hMPV activity occurred mainly during the winter months, peaking in February Y1 and again in October Y2. In Y1 hMPV season lasted from week 47 to 39, but was found predominantly in weeks 1 through 17. There was a small peak in hMPV activity in week 6, and one outlying hMPV case in week 26. In Y2 the hMPV season lasted from week 38 to 10, but occurred predominantly between weeks 38-50.

In comparison RSV and influenza activity was more restricted to the winter periods. RSV appeared from November to April Y1 peaking in December Y1, and appeared again from September Y2 through to March Y2, peaking in November Y2. In Y1 the RSV season lasted from weeks 42 to 17 and peaked in week 49. In Y2 the RSV season lasted from weeks 37 to 13 and peaked in week 47.

Influenza was detected in November through to April 2001/2002, and again in October through to March 2002/2003, peaking in February 2002 and March 2003. In Y1 the Influenza season lasted from week 50 through to 16 and peaked in week 5. and in Y2 from week 43. Very few influenza samples were detected until the second half of the season and was still on the increase by week 12 when the study ended.


Number of Samples .
0
5
10
15
20
25
30
35
40
Week
40
41
42
43
44
45
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10
11
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13
14
15
16
17
19
18
20
21
22
23
24
25
26
27
28
Total Number of Samples
hMPV
RSV
Influenza
RCGP ILI Index
0
5
10
15
20
25
30
35
40
45
50
RCGP ILI index .

A

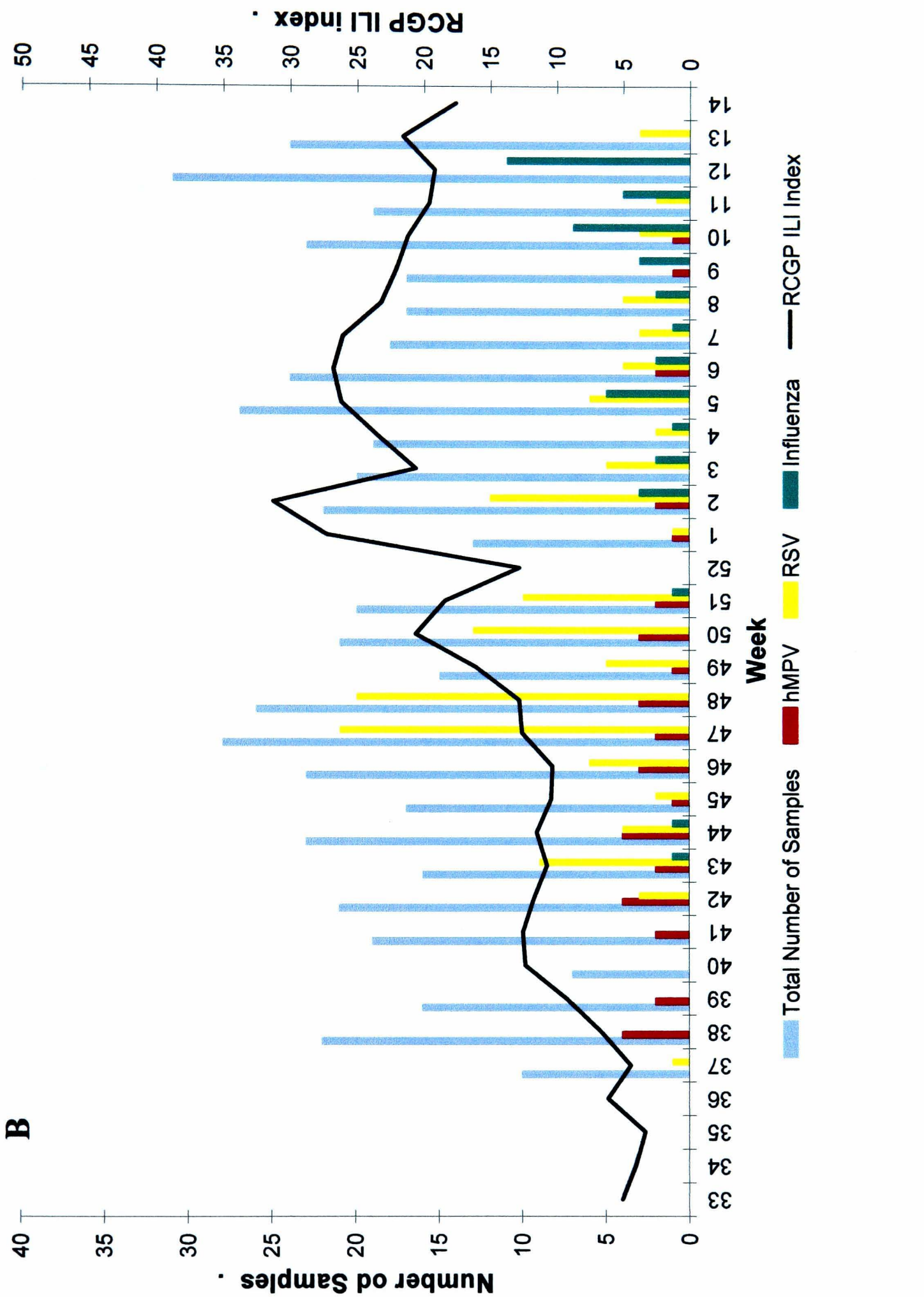


Figure 3.13: Weekly distribution of hMPV, RSV and Influenza from hospitalised children in relation to the ILI index. (A) Year 1 , and (B) Year 2.

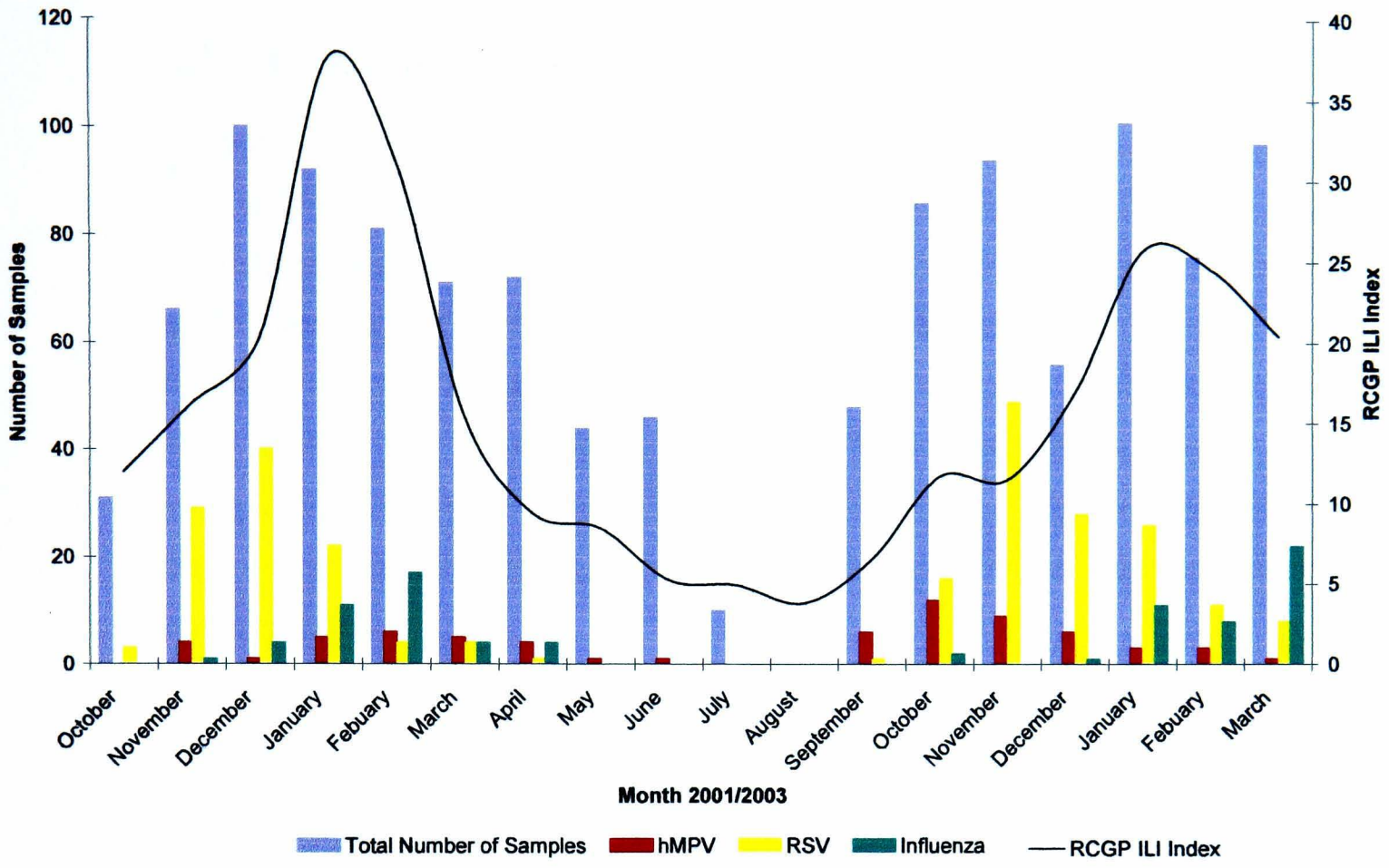


Figure 3.14: Monthly distribution of hMPV, RSV, influenza and total number of samples collected from hospitalised children throughout the 18 moth period of the study related to the RCGP ILI index.

3.7. Community Acquired Infections in the Elderly

A total of 264 clinical samples (combined nose and throat swabs) collected from October 2001 to March 2002 as part of a study investigating the cause of ARTI in the community dwelling elderly were tested prospectively for RSV and influenza, and retrospectively for hMPV using hMPV PCR 1. The total burden of illness caused by hMPV, RSV and Influenza is shown in Table 3.10. In total only 12.5% of the samples were positive for either hMPV, RSV or Influenza, leaving 87.5% without identification of a possible aetiological agent. hMPV accounted for 4.5 % of the total number of samples (36% of those with a virus detected) and was the most commonly detected virus. No dual infections were identified.

Table 3.10: Percentage of hMPV, RSV and influenza cases detected in the community dwelling elderly with ARTI over winter season 2000-2001.

	hMPV %	**RSV %**	**Influenza %**	**Unknown %**	**Total Number of Samples**
% of positive samples	4.5	3.8	4.2	87.5	264
% of Samples with Virus Detected	36.4	30.3	33.3	-	-

The sex distribution of hMPV compared with RSV and influenza is shown in Table 3.12. Overall 56% of the total number of samples were from female patients. One third of hMPV and Influenza infections were in females. In comparison more than two thirds of RSV infections were in females.

Table 3.11: Sex distribution of patients with hMPV, RSV and influenza infections detected in the community dwelling elderly with ARTI over winter season 2000-2001.

Sex	**hMPV**	**RSV**	**Influenza**	**Total**
Number of Males	7	3	6	**91**
% Grand Total	2.7	1.1	2.3	34.7
% of Males Only	7.7	3.3	6.6	100
% Total virus	58.3	30.0	54.5	
Number of Females	4	7	3	**148**
% Grand Total	1.5	2.7	1.1	56.1
% of Females only	2.7	4.7	2.0	100
% Total virus	33.3	70	27.3	
Number Sex Not Known (SNK)	1	0	2	**25**
% Grand Total	0.4	0	0.8	9.5
% SNK only	4.00	0	8.00	100
% Total Virus	8.3	0	18.2	
Total virus	**12**	**10**	**11**	
Grand Total				**264**

The weekly analysis of the distribution of hMPV, RSV and Influenza is shown in Figure 3.16. The season spanned 23 weeks from weeks 44 to 14. hMPV appeared sporadically throughout the winter season from week 46 to week 10 with no apparent peek in incidence. In comparison, RSV was detected in weeks 47 to 6 and peaked in week 1. Influenza was detected in weeks 1 to 10 peaking in week 6.

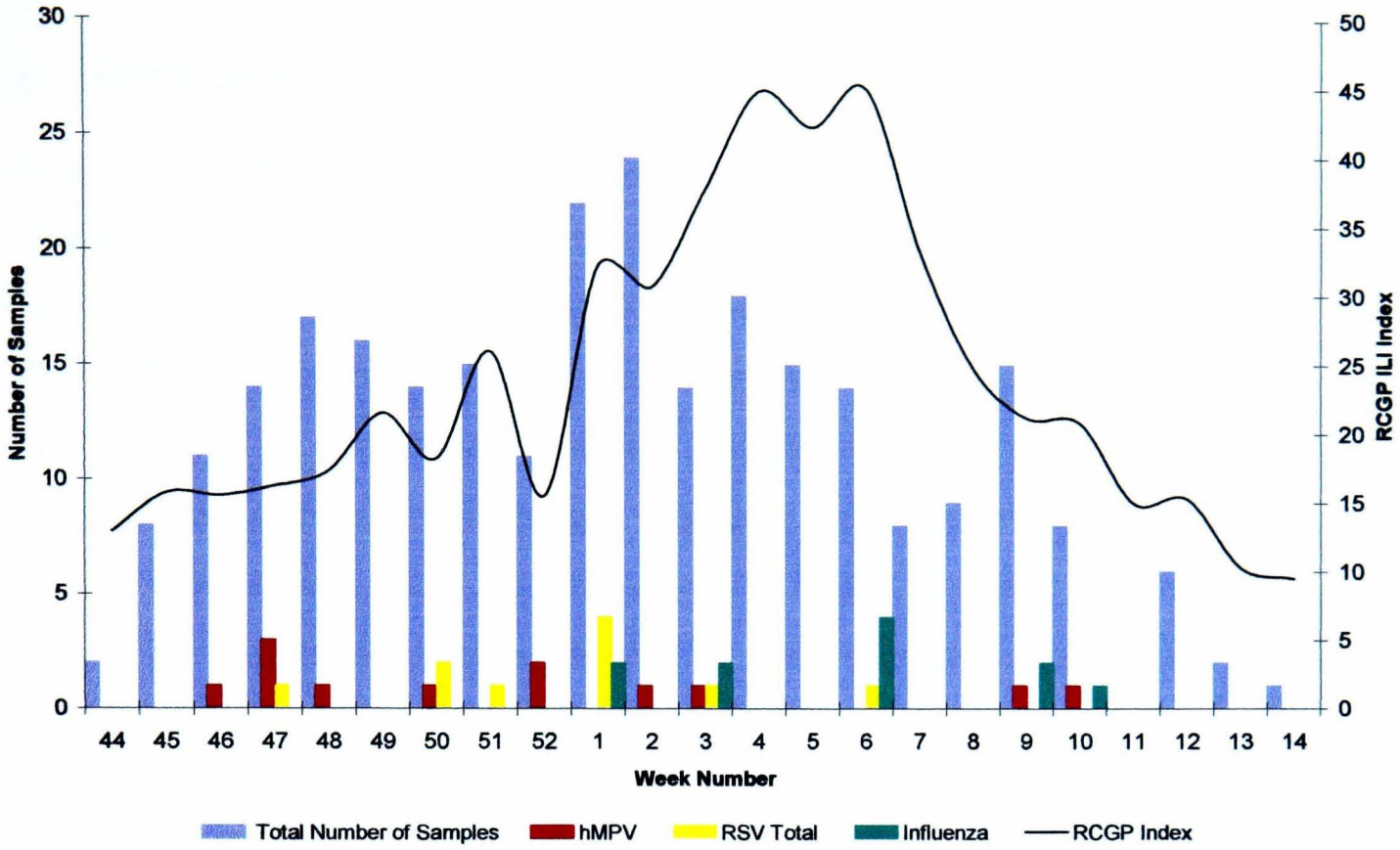


Figure 3.15: Weekly distribution of the total number of samples received and hMPV, RSV, influenza infections detected in the community dwelling elderly with ARTI over winter season 2000-2001.

3.8. Immunocompromised Patients

A total of 134 clinical samples (nasopharyngeal aspirates) collected as part of a study investigating the cause of ARTI in hospitalised immunocompromised patients between November 2002 and March 2004. Samples were tested retrospectively for hMPV using hMPV PCR 2. The patients studied were hospitalised at the Royal Free in London, and covered a range of immunocompromised conditions, the majority of patients having undergone bone marrow transplantation. All other investigations into viral or bacterial pathogens were carried out at the Royal Free Hospital of which no information was available. The total burden of illness for hMPV is shown in table 3.13. hMPV accounted for 8.2% of the total number of samples over the 2 years.

Table 3.12: Percentage of hMPV cases detected in hospitalised immunocompromised patients with ARTI over winter 2 consecutive winter season 2002-2004.

	hMPV	Unknown	Total Number of Samples
No of Samples	11	123	134
% Total No of Samples	8.2	91.8	100

The monthly distribution of hMPV is shown in Figure 3.16. Samples span from November through to March. hMPV was detected in all months with the exception of November with the largest number of cases occurring in December.

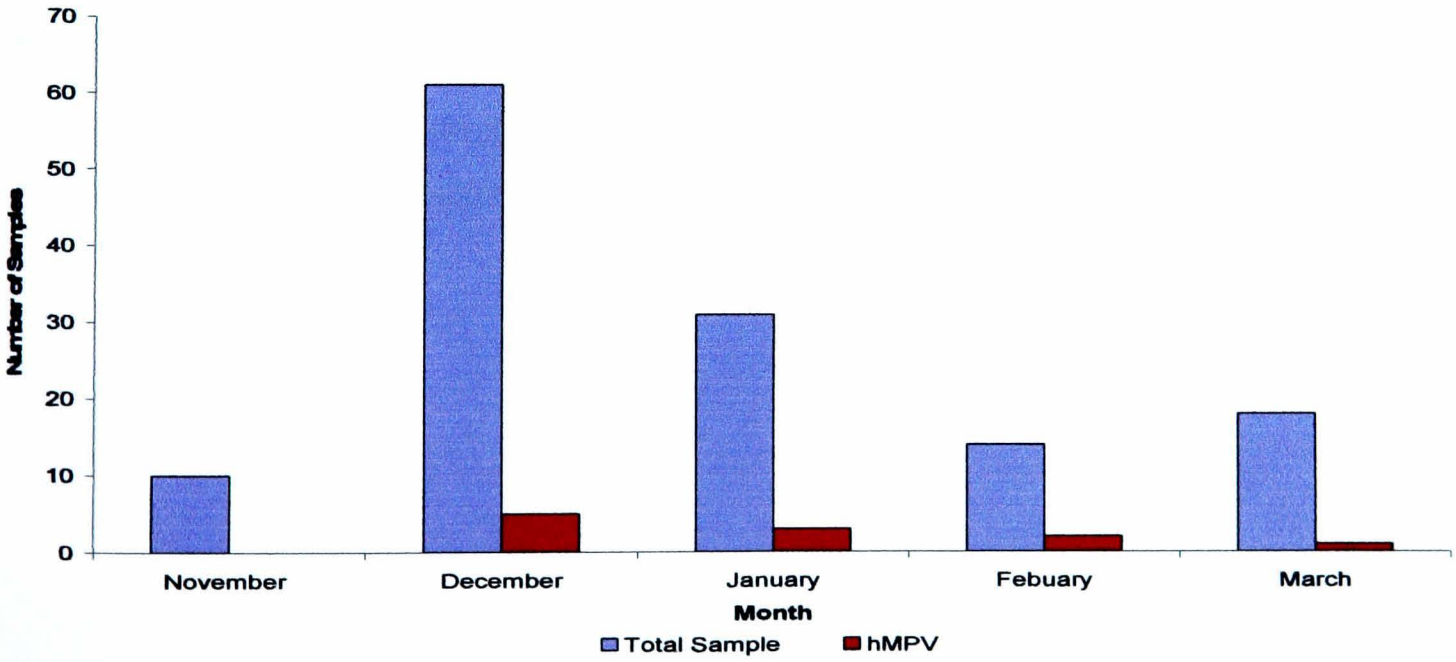


Figure 3.16: The monthly distribution of hMPV cases detected in hospitalised immunocompromised patients with ARTI over winter 2 consecutive winter season 2002-2004.

The age distribution of patients with hMPV infection is shown in Figure 3.17. There were similar numbers of samples for each age group, with the exception of the <1 year age group, which accounted for almost 30% of the samples. As such the largest number of hMPV infections was detected in children less than one year of age (2, 5, 6 and 8 months old) of which, one was a pre-term baby. The highest proportion, however, was in the 15-24 year age group. The older patients with hMPV infections had all received bone marrow transplants.

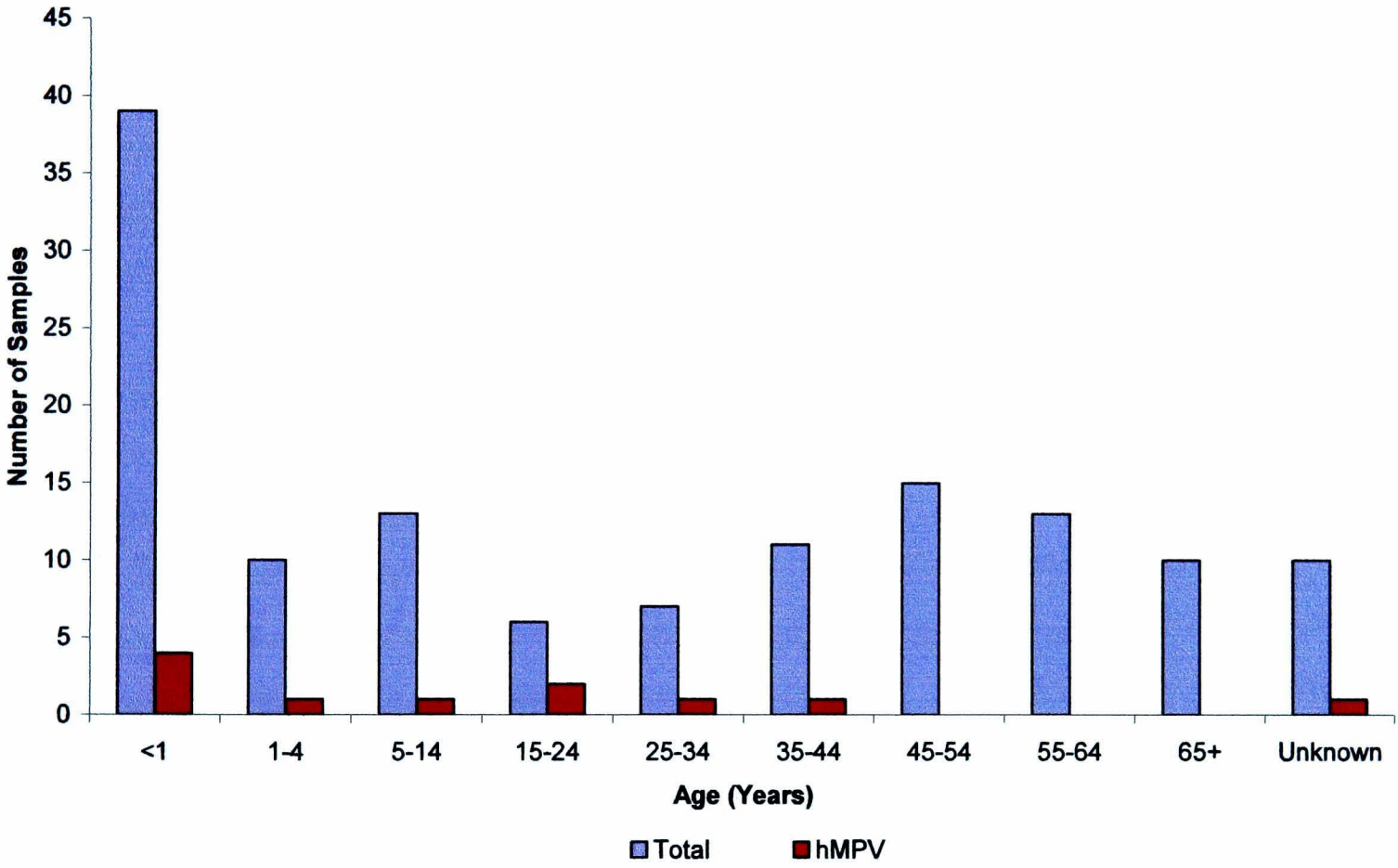


Figure 3.17: The age distribution of hMPV cases detected in hospitalised immunocompromised patients with ARTI over winter 2 consecutive winter season 2002-2004.

3.9. Summary

Table 3.14 summarises the key results by year of the different clinical studies described above.

Year 1 includes 3 studies; community acquired ILI, ARTI in Hospitalised children, and ARTI in community dwelling elderly. The largest proportion of hMPV cases occurred in hospitalised children and the elderly. The largest proportion on hMPV infections in the community occurred in 1-4 year olds. The proportion of RSV in the community was greater than that in the elderly by more than 1½ times. Influenza as expected, was most prevalent in the community ILI study with the smallest proportion was in the elderly cohort, where the uptake of influenza vaccine is considerably larger than the other cohorts investigated.

Year 2 also included 3 studies; community acquired ILI, ARTI in Hospitalised children, and ARTI in Hospitalised immunocompromised patients.. The largest proportion of hMPV cases were identified in patients hospitalised with compromised immunity, followed by hospitalised children. The largest proportion on hMPV infections in the community occurred in 1-4 year olds. No Information regarding the incidence of RSV or influenza was available for the immunocompromised study, however, RSV occurred in roughly equal proportions in both the community and hospitalised children due to the severity of the RSV season. Influenza, consistent with Y1 was most prevalent in the community.

Year 3 included 2 studies; community acquired ILI and ARTI in hospitalised immunocompromised patients. Consistent with Y2 the largest proportion of hMPV infections occurred in patients with compromised immunity and the largest proportion on hMPV infections in the community occurred in 1-4 year olds. There was also a difference in the age groups in which hMPV peaked. In both Y2 and 3 of the immunocompromised study, adults had proportionally the highest number of hMPV infections.

Table 3.13: Summary of finding of the prevalence of hMPV in the different clinical studies investigated.

Year	Study	Number of Samples	hMPV (%)	RSV (%)	Influenza (%)	Other Virus (%)	Dual Infection (%)	No virus Detected (%)	hMPV Peak Age
1 01/02	Community	389	14 (3.6)	24 (6.2)	75 (19.8)	-	0	276 (70.9)	1-4
	Hospitalised children	613	27 (4.4)	103 (16.8)	41 (6.7)	178 (29.0)	33 (5.4)	297 (48.4)	<6
	Community Elderly	264	12 (4.6)	10 (3.8)	11 (4.2)	-	0	231 (87.5)	>65
2 02/03	Community	372	19 (5.11)	94 (25.3)	76 (20.4)	-	11 (2.9)	183 (49.2)	1-4
	Hospitalised children	558	40 (7.2)	139 (24.9)	44 (7.9)	182 (32.6)	57 (10.2)	210 (37.6)	<6
	Hospitalised Immunocompromised Patients	45	5 (11.1)	-	-	-	-	-	25-34
3 03/04	Community	655	35 (5.3)	27 (4.1)	247 (37.7)	-	10 (1.5)	346 (52.8)	1-4
	Hospitalised Immunocompromised Patients	89	6 (6.7)	-	-	-	-	-	15-24

3.10. Discussion

3.10.1. PCR Development

The RT-PCR strategies adopted in this work are based on the detection of the fusion gene of the hMPV genome. hMPV PCR 1 was the original PCR developed at the HPA Colindale when hMPV was first identified; and as such was designed when limited sequences were available. HMPV PCR 2 was developed in this work when a greater number of sequences were available. hMPV PCR 2 was compared with hMPV PCR1 and was found to have a higher specificity and sensitivity, when tested using a cDNA dilution series.

3.10.1.1. Nucleic acid extraction

The extraction method used in this work uses guanidine isothiocynate and guanidine hydrochloride. These are chaotrophic agents which disrupt the virion and cellular material by denaturing proteins, therefore also inhibiting any nuclease activity. Nucleic acid binds to the sepharose surface of magnetic glass beads due to the high chaotrophic salt conditions. Unbound substances including denatured proteins, membrane material and other PCR inhibitors are removed by gradually reducing the salt concentration. Purified nucleic acid is eluted at low salt concentrations and elevated temperatures. This method has been shown to be a sensitive method for extracting nucleic acid from clinical samples, and has been fully optimised and well validated at HPA Colindale and elsewhere for analysis of respiratory samples (Stockton et al., 1998).

3.10.1.2. Reverse transcription

The cDNA synthesis method used in this work is based on random hexamer priming. Random hexamers prime reverse transcription by binding at multiple points along the template. It is a generic approach to generating cDNA and enables a variety of pathogens to detected be subsequently by PCR. Random hexamer priming is also useful for templates with difficult secondary structures or for fragmented RNA. Random hexamers have also shown to improve the transcription of 5' regions more efficiently (Promega, 2006). This method has been fully optimised and validated at HPA Colindale and elsewhere for the analysis of respiratory samples (Stockton et al., 1998). There are drawbacks with using a

random hexamer approach in that it is non specific and all RNA species within the sample will be used as template for first strand synthesis therefore increasing the chances of non-specific amplification by the PCR. Other options for RT priming are Oligo (dT), and gene specific primers. Oligo (dT) are more specific than random hexamers in that they prime at the poly A tail of mRNA species, therefore the complexity of the cDNA is less than with random hexamers. However only mRNA species are targeted, and not all RNA viruses use a poly A tailed message. Therefore Oligo (dT) is not optimal for clinical samples of this type, and may result in decreased sensitivity of the assay. Gene specific primers (GSP) are the most specific method of performing an RT by hybridising at a specific site at the 3' terminus of the mRNA species. They are useful when there is a low abundance of transcripts and may be useful for producing longer cDNA species. However, GSP often fail to prime, and due to the specificity of the primer only the corresponding PCR may then be subsequently used. This is not ideal when a samples requires testing for multiple agents.

3.10.1.3. PCR

In this study over 20 different primers (some of which were previously published)(Table 3.15) were evaluated theoretically using the Oligo 6 primer design software (Molecular Biology Insights, Inc).

Primers were selected based on sequence analysis, the ability to create primer pairs for both single and nested PCR approaches, amplicon length, melting temperature, GC content, primer length, repeats, 3' end stability, primer dimers and hairpins.

This reduced the number of primers to 6 (Table 3.15), which were tested experimentally in all possible combinations as single and nested PCRs against laboratory grown material using the standard PCR conditions with an annealing temperature of 50°C. Products were all of expected size and sequencing confirmed the correct products were obtained. The primers chosen had the greatest specificity in the primary and secondary rounds of the PCR. A nested or hemi nested PCR approach was preferred due to the increased in sensitivity it offers.

Biochemical optimisation of the both the primary and secondary round of the selected PCR was investigated. This included altering the concentration of Tris-HCL, $MgCl_2$, KCl and

the pH. The optimal annealing temperature for each round of the PCR was also determined.

Table 3.14: Complete list of hMPV F gene Primers analysed theoretically using Oligo 6 primer design software (Molecular Biology Insights, Inc).

Footnotes: (i)*Primer positions are relative to reference strain NL00-1 accession number AF371337. (ii) The shaded primers were selected for experimental testing.

Primer	Sequence 5'-3'	Sense	Position*	Source
HMPVF2941F	CTT GGC CAA GAG CTA CTA AC	+	2941-2960	This work
HMPVF3052F	ATG TCT TGG AAA GTG GTG AT	+	3052-3071	This work
HMPV4390 F	GAC CCA GTC AAG TTT CCT GAA	+	4390-4410	This work
HMPVF283F	ATT GAA AAT CCC AGA CAA TC	+	3334-3353	This work
HMPVF4734R	GGA GCC TTG CGA GAC ATT AT	-	4753-4734	This work
HMPVF4651R	TAA TTA TGT GGT ATG AAG CC	-	4670-4651	This work
HMPVF1622R-A	ACT ATG CGG TAT AAA ACC GC	-	4668-4648	This work
HMPVF1622R-B	ATT ATG TGG TAT AAG ACA TT	-	4668-4648	This work
HMPVF1622R-C	CTA ATT ATG TGG TAT GAA GC	-	4671-4652	This work
HMPVF4651R-A	CTA ACT ATG CGG TAT AAA ACC	-	4671-4651	This work
HMPVF4651R-B	CTA ATT ATG TGG TAT GAA GCC	-	4671-4651	This work
HMPVF4737R	GCA CGG AGC CTT GCG AGA CAT	-	4757-4737	This work
HMPVF575R	TCT GTT GAA TTG ACT GAA GC	-	3645-3626	This work
HMPV 1499	GCG GCA ATT TTC AGA CAA CG	+	3663-3682	Nicholson *et al.*, 2006
HMPV 1897	CAG CAG CAG GAA TCA ATG TT	+	4061-4080	Nicholson *et al.*, 2006
HMPV 2175	ACA TGC TGT TCG CCT TCA AC	-	4358-4339	Nicholson *et al.*, 2006
HMPV 2166	TCG CCT TCA ACT TTG CTT AG	-	4349-4330	Nicholson *et al.*, 2006
MPVF1	AGC TGT TCC ATT GGC AGC A	+	4216-4234	Chan *et al.*, 2003
MPVF3	GAG TAG GGA TCA TCA AGC A	+	4238-4256	Chan *et al.*, 2003
MPVF2	ATG CTG TTC RCC YTC AAC TTT	-	4356-4336	Chan *et al.*, 2003
MPVF4	GCT TAG CTG RTA TAC AGT GTT	-	4335-4315	Chan *et al.*, 2003
PeretHMPVF1F	CTT TGG ACT TAA TGA CAG ATG	+	3704-3724	Peret/NEJM SARS
PeretHMPVF1R	GTC TTC CTG TGC TAA CTT TG	-	4153-4134	Peret/NEJM SARS
HMPV-F1	ATG TCT TGG AAA GTG GTG	+	3052-3069	Boivin *et al.*, 2002
HMPV-F2	TCT TCT TAC CAT TGC AC	-	3810-3794	Boivin *et al.*, 2002

3.10.1.4. Sensitivity

Due to the poor growth properties of hMPV in cell culture it was not possible to relate the sensitivity of the PCR to virus infectivity by determining the number of pfu/ml of virus. Therefore in vitro transcripts were made of the target region of the hMPV F gene and applied to the RT-PCR in a dilution series. The PCR detected 1x 10^4 copies of RNA transcript/ reaction and 1x 10^4 copies of plasmid DNA. This appears to be rather insensitive for a nested RT-PCR where 1x10^3 copies of RNA should be easily detectable. The insensitivity of this PCR may be underestimated due to inaccurate calculation of RNA

copy number using a spectrophotometer, or RNA degradation . More sensitive and accurate methods of RNA quantification could be considered for future work. The presence of an additional band in the PCR products generated may also decrease the sensitivity. Further optimisation of buffer conditions, primer concentration, quantity of Taq polymerase and cDNA would also ideally have been investigated and would likely have improved sensitivity. Alternative targets could also have been investigated, however due to time constraints further optimisation was omitted.

3.10.1.5. Specificity

A wide range of other viral and bacterial pathogens may also be present in the clinical samples, along with human chromosomal material. To ascertain whether the presence of such genomes would interfere with the PCR, resulting in non-specific amplification, a number of clinical samples and samples known to contain a variety of these pathogens were tested in the PCR. Sequence analysis of the amplicons was also performed to confirm specificity. There was no cross reactivity observed with any of these, or with the clinical samples, with the exception of some low molecular weight smearing in some samples. All amplicons that were produced were of the expected size and sequence. Further improvements to assay design could be contemplated. With regard to the low molecular weight smearing observed in some samples the use of HOT START *taq* polymerase could be investigated. This utilises an immobilised Taq polymerase. The Taq is therefore inactive until the correct melting temperature is reached and the Taq is released. This prevents amplification of products which may occur before the optimal temperatures are reached, and has been shown to increase the specificity PCR.

Since the initial reports of hMPV detection by RT-PCR a number of other conventional and real time RT-PCR assays have been reported, a summary of which is given in Table 3.16. Some assays have targeted the L, M, or P genes, however a majority have targeted either the F gene, as in this work, or the N gene. Due to the proximity of the N gene to the 3' promoter RT-PCR assays targeting this gene are likely to be more sensitive than those targeting other genes. The F gene was targeted in this work due to the availability of sequence at the time, future PCR development however should target the N gene.
Real time PCR also offers many improvements in the sensitivity of RT-PCR assays with as few as a 1000 copies/ml being readily detected (Kuypers et al., 2005). Real time assays

also offer greater versatility in terms of turn around time, and reduced samples quantities, many also provide quantitative data. Future development of diagnostic PCR for hMPV would therefore ideally be based on a real-time format.

Table 3.15: Summary of reported PCR methodologies for the detection of hMPV. (RT) Real time format, (C) Conventional format

Target Gene	Position	Publication	Format	Comment
N	104-1025	(Boivin et al., 2003)	RT	GSP RT Step
	742-965	(Konig et al., 2004)	C	
	490-807	(Xepapadaki et al., 2004)	C	
	74-245	(Rohde et al., 2005)	RT	
	993-1064	(Sumino et al., 2005)	RT	
	389-1004	(Samransamruajkit et al.,	C	Nested
	556-814	2006)		
	80-596	(Sarasini et al., 2006)	C	
	40-766	(Gerna et al., 2005)	C	
	641-813	(Bouscambert-Duchamp et	RT	
		al., 2005)		
	97-1025	(Cote et al., 2003)	RT	
F	4216-4357	(Chan et al., 2003)	C	Nested
	4238-4335			
	39010-4566	(Esper et al., 2003)	C	
	3704-4153	(Peret et al., 2002)	C	
	3163-3443	(Esper et al., 2004)	C	
	3647-4153	(Samransamruajkit et al.,	C	Hemi nested
	3704-4153	2006)		
	3619-3686	(Kuypers et al., 2005)	RT	A or B subtypes
	or			
	3619-3693			
	3626-4155	(Gerna et al., 2005)	C	
	3626-3767	(Ebihara et al., 2004)	C	
P-M	1864-2185	(Mackay et al., 2004)	C/RT	
	1798-1878	(Agapov et al., 2006)	RT	
M	2630-2830	(Peiris et al., 2003)	C	Detects both hMPV & APV
	2396-2598	(Mackay et al., 2003)	C	
L	11321-11490	(van den Hoogen et al., 2003)	C	Dot blot hybridisation.
	1152-11486	(Samransamruajkit et al.,	C	Hemi nested
	11336-11486	2006)		
	11288-11457	(Sarasini et al., 2006)	C	
	7308-7857	(Cote et al., 2003)	RT	

Other methods used for the detection of hMPV in clinical samples include indirect IF or nasopharyngeal secretions (Ebihara et al., 2005) and immunofluorescence of shell vial cultures (Landry et al., 2005), however both these reports compared the IF assays to RT-PCR and both proved RT-PCR to be more sensitive. A number of serological assays have also been developed for the detection of hMPV antibody as will be discussed in chapter 6.

Whilst these assays are useful tools for the assessment of seroprevalence, and other immunological investigations as well as confirmatory diagnosis if seroconversion in convalescent serum is measured, they cannot be readily used in the acute phase of illness.

3.10.2. Sampling

The optimal sample type for hMPV has not yet been determined. The samples investigated in these studies, nose and throat swabs, and NPAs are the most typical samples for investigating acute respiratory tract infections. However, as hMPV has been shown to cause a broad range of illness other sample types should not be overlooked. Both hMPV and RSV have been found in middle ear effusions, conjunctival brushes, paranasal sinus tissue, BAL and sputum samples to name but a few. (Boivin et al., 2002, Suzuki et al., 2005, van den Hoogen et al., 2003) Recently, hMPV was also detected in the plasma of 41% of patients with hMPV positive nose swabs (Maggi et al., 2003) and recovered from lung and brain material of a fatal encephalitis case of other wise unknown cause (Schildgen et al., 2005). Investigations into the optimal sample types should be carried out. Each sample type, however, has its own bias and it is unlikely one sample type will suffice for all clinical presentations.

Transit of the sample from the GP/ Hospital to the laboratory is also an important consideration. In the community study swabs were put into virus transport medium and sent by first class mail at ambient temperature to the laboratory arriving approximately 3 days from when the sample was taken. It is possible a degree of sample degradation occurs during this time. The samples taken for the hospital study by comparison were couriered to the laboratory the same day, which, may contribute to the increase detection rate of hMPV in this study. Upon receipt, samples were aliquoted immediately, to avoid repeated freeze thawing. PCR aliquots were put in to guanidine based buffers to help preserve the sample. A portion of the original sample, however, was frozen without guanidine and used for virus isolation in cell culture. Repeated freeze thawing or long term storage of this sample may result in sample degradation, and reduce the recovery rates of virus in culture. This is particularly important for hMPV which is already a very fastidious virus to grow, and like RSV is thought to be sensitive to freeze thawing (Gupta et al., 1996).

The majority of hMPV PCR testing was carried out on cDNA previously used for testing for other agents, and may well have undergone several freeze thaw cycles before hMPV testing was carried out. Again resulting in sample degradation which is not optimal for the detection of RNA. Monitoring of sample degradation could be achieved through the analysis by RT-PCR of housekeeping genes such as GAPDH or β-actin.

3.10.3. Epidemiology

hMPV PCRs 1 and 2 were applied to clinical material from a variety of different clinical studies to determine the level of ILI or ARTI within different UK populations that can be attributed to hMPV infection.

It is worth noting that the number of samples received from both the community and hospital study decrease by almost half in week 52, and is reflected in the RCGP ILI index. It is unlikely that this is due to a decrease in RTI during this time and more likely due the closure of GP surgeries, reduction in the postal service, and a reluctance to hospitalise children over the Christmas and New Year holiday. The incidence of hMPV, RSV and Influenza may therefore be under estimated for that period.

Table 3.17 summarises the relevant published data on the detection of hMPV in various studies conducted on samples obtained in different study groups and different seasons. The publications have been grouped according to the main focus of the study population, but is not absolute, and is not intended to be an exhausted list of publications on hMPV epidemiology.

Table 3.16: Summary of published addressing the prevalence and epidemiology of hMPV. NL: Netherlands, UK: United kingdom, BRA: Brazil, USA: United States of America, SPA: Spain, GER: Germany, IND: India, CAN: Canada, JAP: Japan, ARG: Argentina, ITA: Italy, THA: Thailand, AUS: Australia, FL: Finland, CHI: China, HK: Hong Kong,

NOR: Norway, SA: South Africa, DEN: Denmark, GRE: Greece, FRA: France, IRE: Ireland, MEX: Mexico, ISR: Israel. A: Samples tested are negative for other respiratory pathogens. B: Co- infections with hMPV detected, N: A symptomatic or negative control group included. COPD: Cardio Pulmonary Disease, RD: Respiratory Distress, NS: Not Stated. Where spaces are blank, the relevant information was not reported.

Population	Author	Year	Country	Study group	Study period	Number	% hMPV	Comment	Peak age	Peak period
Commuhity	van den Hoogen	2001	NL	< 10 Yr- RTI	20 Years	68	7 (10%)	A	4-74Yr	ND
	Stockton	2002	UK	ILI	Oct 00- Mar 01	408	9 (2.2%)	A	>65Yr	Dec
	Cuevas	2003	BRA	Children- ARTI	May and June 202	111	19 (17%)	B		
	Esper	2003	USA	< 5 Yr- U/LRTI	Oct 2001 to Feb 2002	296	19(6.4%)	A	8.7Mo	Jan- Feb
	Vicente	2003	SPA	<3 Yr-ARTI	Nov 01-Feb 02	147	6(4.1%)		7-20Mo	
	Konig	2004	GER	<3 Yr- ARTI	Nov 99-Oct 01	83	15(18%)	B		
	Mullins	2004	USA	Children-ARTI	Aug 00- Sep 01	668	26(3.9%)		1-2Yr	Mar- Apr
	Rao	2004	IND	ARTI	Jul- Aug 03	26	15(19.2%)			
	Williams	2004	USA	Children -LRTI	25 years	248	49(20%)	N	11-12Mo	Dec-Apr
	Chano	2005	CAN	Children-RTI	Winter 01	1132	44(3.9%)	BN		Jan- Feb
	Sasaki	2005	JAP	children-ILI	02-03 & 03-04 RS	1498	84 (5.6%)			Feb
	Falsey	2006	USA	>18 years - +/- RTI		164(+RTI) & 158 (- RTI)	(+RTI) & 0(0%) (- RTI)	N		winter
	Williams	2006	USA	Children- URTI	82-01 (20 years)	2384	18(5%)	A		Dec- May
Community	Galiano	2004	ARG	<5 Yr-ARTI(bronchiolitis)	98-02	100	11 (11%)	A		Spring
&	Bosis	2005	ITA	<15 Yr - non respiratory	Nov 02- Mar 03	1505	42 (2.8)	B		winter
Hospital	Kuypers	2005	USA	ARTI	Dec 02-May 03	719	52(7.2%)		7-12Mo	Apr- May
	Robinson	2005	CAN	children-ARTI	Nov 02 - Dec 03	1079	42(3.9%)			Jan- Apr
	Agapov	2006	USA	Children	Mar 02- June 04	3740	202 (5.4%)	A	<2Yr	Feb - Apr
	Kaida	2006	JAP	<3 years - RTI- Encephalitus	Jun 04-May 05	144	29(20.1%)			Apr
	Saramaronsmurajit	2006	THA	Children- LRTI	Mar 01- Nov 03	220	12 (5.4%)			
Hospital	Howe	2002	AUS	Children- ARTI	NS	525	40(7.6%)	A		
	Jartti	2002	FI	3mo - 16 y Expiratory Wheezing	Sep 00-May 01	132	10 (8%)	B		
	Bastien	2003	CAN	ARTI	Oct 01-Apr 02	445	66(14.8%)	A	<5 years>50years	
	Boivin	2003	CAN	<3 Yr- ARTI	Dec01-Apr 02	208	12 (6%)		<2yYr	Mar- May
	Chan	2003	CHI	SARS	Mar-03	48	25(52.1%)	SARS		
	Falsey	2003	USA	ARTI/ COPD	Winter 99-01	984	44(4.5%)	N	Young adults	Feb
	Freymouth	2003	FRA	Children- ARTI	Winter 01-02	337	19(6.6%)	A	3Mo-12Yr	Dec- Jan
	Kuiken	2003	CHI	SARS	Mar-03	335	41(12%)	SARS		
	Maggi	2003	ITA	Infants- ARTI	Winter 00-02	75 & 15	21(28%) & 2(3%)	B	7.8Mo	Jan- Jun
	Peiris	2003	HK	<18 Yr-ARTI	Aug 01-Mar 02	581	32(5.5%)	B	3-72 Mo	Sring
	van den Hoogen	2003	NL	ARTI	Sep 00-Feb 02	681	47(7%)	A	4-6Mo	Dec-Jan
	Viazov	2003	GER	< 2Yr- U/LRTI; Wheezing, RD	Jan - May 02	63	11(17.5%)	B		Jan- Apr
	Dollner	2004	NOR	Children- ARTI	Nov 02-Apr 03	236	50 (21%)			Nov - Jan
	Esper	2004	USA	<5Yr-ARTI	Nov 01-Oct 02	668	54(8.1%)	A		March
	FF	2004	SA	Children -ARTI		137	18(5.8%)			
	Jartti	2004	FIN	3mo- 16 Yr- Expiratory wheezing	Sep 00-May 02	132	10(8%)	B		Epidemic 2001
	Kashiwa	2004	JAP	children-LRTI	Sep 02-Aug 03	27	16(59%)			Apr -Jul
	Smuts	2004	SA	<3 Yr- ARTI	Apr-Aug 01 & 02	143	16(11.2%)	B		Winter
	von Listow	2004	DEN	ARTI	99-00 & 00-01 (Nov-May)	383	11(2.9%)	B		
	Xepapadaki	2004	GRE	Acute bronchiolitis	Nov - May (NS)	56	9(16.1%)	B		Feb - Apr
	Bouscambert	2005	FRA	<24months- bronchiolitis	Sep 01-Jun 02	94	8(9%)	B		Jan
	Carr	2005	IRE	48-72 Yr-RTI	Sep 03- Mar 04	168	4(2.4%)	A		
	Ebinhara	2005	JAP	Children- RTI	Apr - May 04	48	11 (22%)	A		
	Noyola	2005	MEX	<3 Yr-RTI	03-04 RS	323	34(10.5%)	A		Feb - Mar
	Rohde	2005	GER	COPD	Oct 99- Jun 04	130	3(2.3%)			
	Williams	2005	USA	>18 years-Asthma exacerbations	Dec 99-Dec 03	101	7 (6.9%)			Jan- May
	Jacques	2006	FRA	Wheezing	Sep 01-Jun 02	192	8(4%)			
	Martinello	2006	USA	Adults-COPD exacerbation	Dec 02 -May 03	50	6(12%)			
	Regev	2006	ISR	<5 Yr - ARTI		388	42(10.8%)	A		Feb - Apr
	Sarasini	2006	ITA	Children - ARTI	Winter & Spring 03-04	306	40(13.1%)			Feb - Mar
Immunocomp	Madhi	2003	SA	Infants- +/-HIV; ARTI	NS	196	14(7.1%)			
	Kumar	2005	CAN	ARTI- Lung transplant	2001-2003	50	1 (2%)	N		
	Gerna	2006	ITA	U/LRTI -lung transplant	Nov 01-May 04	128	60%	BN		
	McAdam	2004	USA	<18 Yr -ARTI	00-02	1257	58(4.6%)	B	3-24 Mo	Jan - Feb
	Larcher	2005	AUS	Children- ARTI	Sep 03-Mar 04	265	43 (16.2%)			Outbreak- Mar
	Martino	2005	SPA	Adults-U/LRTI	Sep 99-Oct 03	512	19(4.6%)			
	Rovida	2005	ITA	ARTI	Winter 01-02	208	12 (5.8%)	B		

3.10.3.1. Community Acquired Infections

Combined nose and throat swabs submitted as part of the national surveillance program for ILI were tested prospectively for Influenza and RSV, and retrospectively for hMPV. Samples were taken between October and March for 3 successive seasons, 2001-2002 (Year 1), 2002-2003 (Year 2) and 2003-2004 (Year 3).

hMPV was detected in all three winter seasons and over the 3 years was found in approximately 4.8% of the samples tested. There was an increase in the detection rate of hMPV between year 1 and 2 from 3.6% to 5.1%, most likely due to the introduction of hMPV PCR 2 in the second year. The incidence of hMPV in years 2 and 3 of the study were around the same level with roughly 5.2% of the patients having detectable levels of hMPV.

In comparison, the incidence of RSV varied greatly over the 3 winter seasons, from 25.3% in Y2 to 4.12% in Y3. In year 3, the incidence of hMPV was greater than that of RSV (5.34% vs. 4.12%). In contrast, the level of influenza activity was similar for years 1 and 2 of the study. However, in year 3 the incidence of influenza almost doubled accounting for over 37% of patient samples. The increase in the incidence of influenza was reflected in the RCGP ILI index, and accounts for the increased number of samples received that year.

The detection rate reported for hMPV in the general community in other publications varies considerably from 2.5% to 20%. This reflects differences in the target population, illness, the length and timing of the studies and the relative sensitivities of the detection assays. The only other study which addresses the contribution of hMPV in community acquired ILI only targeted children in which 5.6% were positive for hMPV over two consecutive winters in Japan.

Other community based studies targeted a broader range of respiratory illnesses i.e. ARTI, URTI, LRTI or RTI. Very few studies have targeted adults, or all ages of the population, and have focused mainly instead on the incidence of hMPV in children. One study which did address the incidence of hMPV in adults > 18 years of age with RTI showed hMPV was present in 3.4% of cases with RTI and 0% of healthy adults (Falsey et al., 2006). One

study which investigated the incidence of hMPV in U/LRTI in children showed hMPV was present in 5 and 12% of cases respectively(Williams et al., 2004, Williams et al., 2006).

The age distribution of samples received reflected the normal age distribution of the population tested. hMPV was detected in all ages of the community in roughly equal numbers. Proportionally, to the total number of samples received, hMPV was most prevalent in the 1-4 year age group, as was observed for RSV. Influenza was also detected in all ages and was most prevalent in school age children of 5-14 years old. These finding supports published data (
Table 3.16). This data however shows that hMPV has the ability to cause ILI in adults leading to the requirement for medical consultation.

Samples received from the RCGP community all ages study were taken only from patients presenting with influenza like illness. The overall incidence of hMPV in the community is therefore undoubtedly underestimated in this study. While it has been shown in this work and that of others that hMPV can cause influenza like illness in children and adults (Nicholson et al., 2006, Sasaki et al., 2005, Stockton et al., 2002), a majority of healthy adults with hMPV infections are likely to have much milder symptoms (Boivin et al., 2002, van den Hoogen et al., 2003). Furthermore, most adults with heavy cold or influenza like illnesses are less likely to attend the GP than children and infants. Therefore a large proportion of the general community with ARTI and ARTI's in general are underrepresented. To fully evaluate the burden of disease caused by hMPV in the community samples from patients with a much broader clinical picture need to be analysed.

3.10.3.2. Community Acquired Infections in the Elderly

Combined nose and throat swabs collected by GPs between October 2001 to March 2002 as part of a study investigating the cause of ARTI in the community dwelling elderly were tested prospectively for RSV and influenza, and retrospectively for hMPV.

hMPV was present in 4.5% of the cases investigated in the elderly study. Although the number of hMPV, RSV and influenza infections were roughly the same, it is interesting that while hMPV accounted for a larger proportion of infections in the elderly that year,

compared with the general community, the proportion of RSV and Influenza were considerably lower. While it was expected that few influenza cases were detected due to the higher uptake of the influenza vaccine in this age group (Fleming et al., 2005) compared with the mean age of the community study, it is surprising that a larger incidence of RSV was not seen. This study highlights the important role hMPV plays in RTI in the community dwelling elderly and is consistent with a findings from a number of other reports that the elderly adults have an increased susceptibility to hMPV infection than younger adults (Falsey et al., 2003, van den Hoogen et al., 2003). Increased susceptibility to hMPV in this age group, may be attributed to waning immunity and increased incidence of underlying diseases such as COPD, may leave them more prone to infection. However the large proportion of cases in which these three viruses were not detected, is significant, and analysis of other viral and bacterial pathogens is required to fill this gap in our knowledge.

3.10.3.3. Hospitalised Paediatrics

Samples collected from children <5 years of age hospitalised with ARTI were analysed for a range of common respiratory tract infections including hMPV. Over the 18 months of the study hMPV was detected in 5.7% of samples, which is slightly higher than the level of hMPV observed in the community and supports findings that children <5 years old are more susceptible to infection with hMPV than other age groups. As expected, RSV was the most frequently identified viral agent. There was an increase in the incidence of hMPV from 5% to 6.6% over the two winter seasons studied, and is again most likely due to the introduction of hMPV PCR 2 in the summer of 2003. Unfortunately the clinical data was not available for analysis, therefore comments cannot be made on the severity of the illness of children with hMPV versus RSV infections, however previously published work suggests that hMPV and RSV infections are clinically indistinguishable. hMPV, therefore, contributes significantly to the number of young children hospitalised each year with ARTI. These results are comparable with those from published findings (

Table 3.16).

The incidence of hMPV in other studies varies considerably from 2.9-22% which is again dependent on the specific target groups, study size, timing and length of study, clinical picture and relative sensitivities of the detection assay used. Again most publications focus on children. Esper *et al* reported the presence of hMPV in 8.1% of samples obtained from children <5 years hospitalised with ARTI in the USA that were negative for other viral pathogens (Esper et al., 2004). Another report found hMPV in 10.8% of children < 5 years hospitalised with ARTI in Israel that were negative for other viral pathogens (Regev et al., 2006). In studies that looked at children < 3 years of age hospitalised with ARTI, hMPV was present in 11.2% of cases in South Africa (Smuts et al., 2004), 10.5% in Mexico (Noyola et al., 2005), and 6% in Canada with a greater percentage of cases occurring in children < 2 years of age (Boivin et al., 2003). In Hong Kong hMPV was present in 5.5% of samples from children < 18 years hospitalised with ARTI, however the incidence of hMPV peaked in children between the ages of 3 – 72 months (Peiris et al., 2003).

3.10.3.4. Immunocompromised

The incidence of hMPV in the immunocompromised study accounted for 8.2% of the total number of samples received. This cohort had the highest percentage of hMPV of all the cohorts examined in this thesis, and supports evidence of a higher rate of hMPV activity in people with compromised immunity. Most of the samples received and hence hMPV positive cases were from children < 1 year old. hMPV was also identified in adults, all of which received bone marrow transplants. However it is also worth bearing in mind that this study encompass patients with a broader spectrum of respiratory illness not considered in the other cohorts, ranging from mild sniffles to severe bronchiolitis. As these patients were all hospitalised at the time of illness it is likely a majority of these infections were nosocomial, and highlight the importance of infection control measures especially in the immunocompromised wards. Unfortunately, there was no information available regarding the incidences of other pathogens, or clinical outcomes.

A number of other reports have focused on, or addressed the question of hMPV in the immunocompromised population (
Table 3.16). One such study compared the incidence of hMPV in RTI of otherwise healthy children, lung transplant recipients and immunocompromised children. hMPV was present in 14%, 25% and 2% respectively (Larcher et al., 2005). Surprisingly however in infants

hospitalised with RTI that were either HIV positive or negative, hMPV was detected in 9.1% of HIV negative children but only 3.7% of HIV positive children. The authors however suggest this may be due to the greater importance of other pathogens in HIV infected individuals such as *P. jiroveci* which accounts for 45% of cases. Other respiratory viruses such as RSV, influenza and parainfluenza, have also been reported to occur less frequently in LRTI of HIV positive children compared to HIV negative children. However the relative risk of hospitalization in HIV positive children with respiratory viruses is much greater (Madhi et al., 2003).

3.10.4. Dual Infections

In the RCGP community acquired and hospitalised paediatric studies dual infections were identified in 1.5% and 7.5% of the total number of samples respectively. There were no dual infections identified in the elderly or immunocompromised cohorts. As the hospital study investigated a larger variety of viral pathogens the increase in the rate of dual infection was expected. Overall, dual infections with hMPV accounted for 85% of all dual infections in the RCGP community, and 26% in hospitalised paediatrics. The percentage of hMPV dual infections alters considerably from 42% of all hMPV infections identified in year 2 to 28 % in year 3 of the RCGP community study. No dual infections were identified in year 1. Of the hospitalised paediatric hMPV cases 34% were dual infections. In both incidences the main co-pathogen of hMPV infections was RSV. The largest number of dual infections in the hospital setting were with RSV and either Rhinovirus or other Enteroviruses the three most commonly identified viruses in that study.

From this investigation it is not possible to determine if both viruses identified in a dual infection are causing disease, or to what extent each virus is contributing to the disease symptoms. It is likely, however, that some symptoms are due to the presence of both viruses. It has been reported that children hospitalised with RSV infections are 10 times more likely to require ventilation/ mechanical support if hMPV is also present (Greensill et al., 2003), suggesting hMPV plays an important role as a co-pathogen. It would therefore be beneficial to investigate whether symptoms associated with dual hMPV infections are more severe or longer lasting than single hMPV infections.

Furthermore, it is not known for how long after infection the PCR is capable of detecting viral nucleic acid or for how long hMPV may persist. It has been reported that RSV has the ability to cause persistent infections which are associated with chronic wheezing and asthma in animal models (Bramley et al., 1999, Dakhama et al., 1997, Hegele et al., 1994) . Recently, Alvarez and Trip *et al* have demonstrated hMPV persistence in mice. In that study genomic RNA was detected in the lungs of BALB/c mice 180 days post infection, with recoverable virus up to 60 days post infection despite the presence of neutralising antibodies and cytotoxic T cells (Alvarez et al., 2004, Alvarez & Tripp, 2005).Whether hMPV can cause persistent infections in humans is yet to be investigated. However, if found to do so, the detection of genomic RNA may be possible long after the symptomatic phase of illness, and may not be associated with the ARTI under investigation.

Further investigation of these points requires volunteer studies with sampling before, during, and after the symptomatic phase of illness, with detailed information as to the clinical picture and outcome.

3.10.5. Seasonality

The start and length of the hMPV seasons differed slightly over the 3 years of the community study. In year 1 hMPV was detected in November through to February, and in years 2 and 3 in October through to the end of March with peak incidence in December or January. The RCGP community studies are designed to target influenza/ RSV activity which have well defined seasonal patterns and samples were therefore only collected over the winter months. Consequently this does not give the scope for determining the amount of hMPV activity over the summer period.

The hospitalised paediatric study, in comparison, looks at samples collected over 18 consecutive months encompassing 2 winter seasons and one summer. The number of samples collected shows an increase in ARTI during the winter months but remains high through the summer with the exception of July and August when few/no samples were collected. hMPV appears from November through to June with a peak incidence in January/February. hMPV then reappears again in September through to March when the study ended, with a peak incidence in October, preceding the peek incidence in the community acquired study for that same year by a couple of months.

hMPV appears to have a seasonal pattern occurring primarily in the winter months, and therefore co–circulates with other seasonal respiratory viruses such as RSV and Influenza. Unlike RSV and influenza, hMPV continues to circulate throughout spring and early summer, and then reappears in late summer early autumn before the start of the RSV season. This is consistent with all the data produced about the seasonality of hMPV in temperate climates (

Table 3.16)

3.10.6. Other Comments

The lack of negative (asymptomatic) control groups highlights one of the inherent flaws of these types of studies, as the incidence of asymptomatic infection is not measured. It is important to consider asymptomatic infections as any virus being shed may be a source of symptomatic infection in other individuals. With the exception of one study in which there were roughly equal proportions of hMPV positive young adults with or without RTI (Falsey et al., 2003), little or no hMPV activity is reported to occur in asymptomatic control groups (Gerna et al., 2006, Williams et al., 2004). These studies however are limited and further investigation is required.

It is also important to note that in a large proportion of samples, especially in the elderly study (87.5%), an aetiological agent was not identified. As most of these studies only examined in incidence of hMPV, RSV and Influenza, there is obvious scope for a much broader range or respiratory pathogens, both viral and bacterial, to be investigated. It should also not be forgotten that a number of so far unidentified pathogens may be responsible for a proportion of the ARTI observed and this undoubtedly deserves further attention..

The data presented in this chapter raises a number of questions relating to strain diversity and the potential of hMPV to cause repeated infections, as seen with RSV, and whether strains circulating in the paediatric population the same as those in older children and adults. Chapter 4 aims to address these points through sequence and phylogenetic analysis of the hMPV G protein. Serological analysis of the prevalence of hMPV antibodies in different populations or age groups would provide valuable information as the prevalence

of hMPV antibodies in these different groups, and their potential susceptibility to hMPV infection, especially when addressing the question of repeated infection. Presented in chapters 5 and 6 is the development of such an assay and its application to try and address some of these questions.

Chapter 4

Analysis of Strain Diversity

4.1 Introduction

One of the important issues surrounding hMPV, as has been shown in chapter 3, and investigated further in chapter 6, is the ability of the virus to infect very young children, possibly at a time when maternal antibodies should be protective, as well as its ability to cause repeated infections throughout ones lifetime. This is similar to hRSV where incomplete immunity to the virus, and or genetic heterogeneity contributes to the virus's ability to evade host immune responses (Melero et al., 1997, Sullender et al., 1998, Zlateva et al., 2005) It is therefore important to develop a thorough understanding of the extent of the genetic and antigenic variability in the different hMPV surface proteins, and to relate this to information regarding the timing of primary infection and re-infection relative to antibody levels.

Phylogenetic analysis of the M, N, P, F, G and L genes of hMPV show that there are two lineages of hMPV; A and B, each with at least 2 sub-lineages (A1, A2 and B1, B2) (Bastien et al., 2003, Mackay et al., 2004). It has recently been reported that an additional A sub-lineage (A3) may exist (Huck et al., 2006).

For RSV and other pneumoviruses the fusion (F) and attachment glycoprotein (G) are the major antigenic determinants, against which neutralizing antibodies are generated.

The G protein of hMPV is highly divergent with approximately 33% amino acid homology reported between lineages (Bastien et al., 2004, Boivin et al., 2004, Ishiguro et al., 2004). The high serine and threonine content of the G protein is also another important characteristic and indicates that like the G protein of hRSV the hMPV G protein is also likely to be highly O-glycosylated (van den Hoogen et al., 2002). These characteristics may aid the virus in evading pre-existing immunity by modifying epitopes. In contrast to G the F protein has been shown to be highly conserved showing 93-100% amino acid homology between lineages (Bastien et al., 2003, Boivin et al., 2004, Skiadopoulos et al., 2004). Analysis of the G protein would therefore provide more valuable information as to the diversity of the circulating stains and immune evasion strategies.

Mutations in a protein may be brought about by nucleotide substitutions, insertions or deletions in its encoding gene, of which the two latter result in frame shift mutations. Frame shift may alter the appearance of the protein dramatically, and may result in premature termination. Nucleotide substitutions may or may not alter the amino acid that is

encoded, or introduce stop codons. Where amino acid changes are brought about the nature of the amino acid substitution, its location within the protein and the involvement of that amino acid in the protein structure or function influences whether the mutation is detrimental or beneficial to the protein or has no effect.

Due to the dependence of RNA virus replication on RNA dependent RNA polymerase, which lacks proof reading capabilities, mistakes/ mutations introduced during replication cannot be corrected (Castro et al., 2005, Drake, 1993, Drake, 1999). Any mutations that are incorporated are passed on to progeny virus, and a mixed population of viruses, a quasispecies, is generated. The existence of quasispecies needs to be considered when designing a sequencing strategy for RNA viruses, and the extent of variation within a viral population can be addressed through the sequence analysis of clones. In general the majority species present in a sample is considered the strain type.

The use of DNA sequencing to estimate the strain diversity of RNA viruses suffers from inherent complications due to enzyme induced errors. A comparison of 3 RNA amplification methods used for DNA sequencing showed that the use of MMLV reverse transcriptase followed by a nested PCR with *Taq* polymerase gave the least errors when compared to a combined RT-PCR using a *Tth* enzyme or a nucleic acid sequenced based amplification (NASBA) method using AMV reverse transcriptase and T7 RNA polymerase. Furthermore this study showed that sequencing of cloned products also reduced error rates (Chadwick et al., 1998).

Phylogenetic analysis is only as good as the alignment upon which it is based. Multiple alignments are generated in three major steps. The first step is a pair-wise alignment in which each sequence is aligned with each of the other sequences in the dataset, starting at one end, and attempts to match all possible pairs using a scoring system such as a DNA or protein weight matrix. The Gonnet 250 protein weight matrix used in this work assigns a score depending on the amino acids present in each position of the alignment. Where identical amino acids are aligned the highest score is given, and where biologically distinct amino acids are aligned a low score is given. Other alignment parameters to be set include a gap opening penalty, which gives a negative score, where insertions and deletions occur resulting in the introduction of gaps in the alignment, and gap extension penalties for increasing the length of the gap. The alignment with the highest overall score has the

maximum likelihood of occurring and is used to construct a guide tree (step 2), from which the process is repeated (step 3).

Sequence alignment and phylogenetic analysis of amino acid sequences is generally considered to be more biologically relevant when dealing with coding sequences, as is the case in this work. However it provides little information as to the changes occurring at the nucleotide level. Direct alignment of the nucleotide sequence also has an inherent flaw as gaps in the alignment are introduced where individual nucleotide bases have been inserted or deleted, therefore, translation of the consensus will result in an amino acid sequence that does not reflect the original sequence. In-frame nucleotide alignments however overcome these problems.

In-frame nucleotide alignments are based on amino acid alignments which are utilised by software such as codon align to align the corresponding nucleotide sequences, introducing gaps of three nucleotides where amino acids have been inserted or deleted, therefore maintaining the open reading frame. Translation of the consensus sequence generated by this process results in an amino acid sequence which represents that of the original.

There are three general ways of performing phylogenetic analysis once an alignment has been generated, maximum parsimony, distance and maximum likelihood. The maximum parsimony method produces phylogenetic trees which minimise the number of steps (mutations) required to generate sequence B from sequence A, and considers every position within the alignment. As such this method is highly time consuming and suitable for only a small number of sequences, or where there is a high degree of sequence homology.

There are a number of available distance methods that can be used, the most common being the neighbour joining method. This method identifies the sequence pair within the alignment that has the smallest number of changes between them, and places them as neighbours on the tree then moves on to find the next closest sequence. This method has the advantage of being fast and is capable of dealing with a large data set. However it is biased by the most closely related sequences, and assumes equal rates of evolution are occurring throughout the sequence and between taxa (the molecular clock hypothesis). As such this method my give rise to an inaccurate account of evolutionary history.

The maximum likelihood method however allows for variation in rates of change and is therefore more suited for more distantly related or highly variable sequences, as is the case in this work. The maximum likelihood approach is similar to that of the maximum parsimony, in that it evaluates all positions within the alignment, and finds the tree with the greatest likelihood based on a model of evolution, and therefore has the draw back of being highly time consuming and computorally intense. The most appropriate evolutionary model for the data set under investigation was determined in this work using the model test application within the PAUP phylogenetic software. Model test analyses the dataset and selects a model which most closely represents the data under investigation from approximately 60 defined models, and provides estimates on the rates of substitution, transition, translation, additions and deletions, which are used as parameters in the phylogenetic analysis.

A number of questions arise from the epidemiological data presented in chapter 3. What is the genetic diversity of the circulating hMPV strains? Do the same strains circulate in community and hospital populations, and at different time points? And what evidence is there that genetic diversity in the attachment protein contributes to antigenic changes and evasion from host immunity? This chapter aims to address these questions through the use of sequence and phylogenetic analysis of the highly divergent G gene of hMPV.

4.2 Aims

- To develop a sequencing strategy for the full length open reading frame of the hMPV attachment glycoprotein and apply it to clinical material in which hMPV has been detected using the F gene diagnostic PCR described in chapter 3.

- To analyse the sequence data to determine the relationships of the hMPV strains circulating.

- To analyse diversity between strains and formulate hypotheses about the functional properties of the hMPV G protein.

4.3 Development of a Sequencing Strategy for the hMPV G ORF

4.3.1 Gene amplification

Primers were designed to amplify the entire length of the hMPV G gene. To avoid regions where large amounts of variation occurred and the need for extensively degenerate primers, forward primers were place primarily in the SH-G inter-genic and G start regions, and the reverse primers placed primarily in the large polymerase gene.

Table 4.1 Primers investigated for use in the amplification of hMPV G ORF from clinical samples

	Primer	Position*	Sense	Sequence
1	HMPVSH5479 F	5479-5498	+	GTG GGA TAA ATG ACA ATG AT
2	HMPVSHABF	5481-5500	+	GGG ATA ART GAC AAT GAW AA
3	MPVSH1	5494-5511	+	ATG AIA TCA TTA GAD AYV
4	HMPVG6247F	6247-6266	+	ATG GAG GTG AAA GTG GAG AA
5	HMPVGABF	6233-6259	+	GGG ACA AGT RGY YAT GGA RGT RAR AG
6	HMPVGAF	6233-6259	+	GGG ACA AGT AGT TAT GGA GGT GAA AG
7	HMPVGBF	6233-6259	+	GGG ACA AGT GGC CAT GGA AGT AAG AG
8	MPVG1F	6235-6258	+	GAG CAA GAA GTA ATG AAC RTG VIA
9	MPVG5F	6242-6269	+	TAG TTA TGI ASA TGA AAG TGA TAR AMA W
10	HMPVG3F	6423-6445	+	ATC TGA ATC AGA ACC TCM CAC MA
11	HMPVG6100F	6100-6120	+	TTT AAA AAT ATT TTG AAA AC
12	HMPVG6260F	6260-6280	+	TGG AGA ACA TTC GAA CAA TA
13	HMPVG257F	6503-6523	+	ACA CCA ACT CAA GCC CAC AGC
14	HMPVG481F	6727-6747	+	CAT TCT CCA CCA CGG GCA ACG
15	HMPVG277R	6503-6523	-	GCT GTG GGC TTG AGT TGG TGT
16	HMPVG501R	6727-6747	-	CGT TGC CCG TGG TGG AGA ATG
17	HMPVG7056R	7056-7036	-	ATG GTG TTG TTG CCT TTT TG
18	HMPVG6686R	6686-6666	-	GCC GGA CTT GTC TTT GTT CT
19	HMPVG7085R	7104-7085	-	TGG TAT ATG GGC GAG TGT TT
20	HMPVLABR	7185-7204	-	AGA TAG ACA TTA ACA GTG GA
21	MPVGLRV1	7167-7192	-	CAG TGG ATT CAT TAA GIG GRT CSA T
22	MPVGLRV2	7205-7227	-	CAT CTT TAA GTT ATA ARA GTC GWA GRY Y

Footnotes: (*) Primer positions based on reference strain NL00-1. Schematic representation of primer positions is shown in Figure 4.1.

Primers were tested in various combinations of single, nested and hemi nested PCR approaches on hMPV positive control material grown in the laboratory using Taq DNA polymerase. Over 50 different combinations were tested many generating products of the incorrect size or non-specific bands.

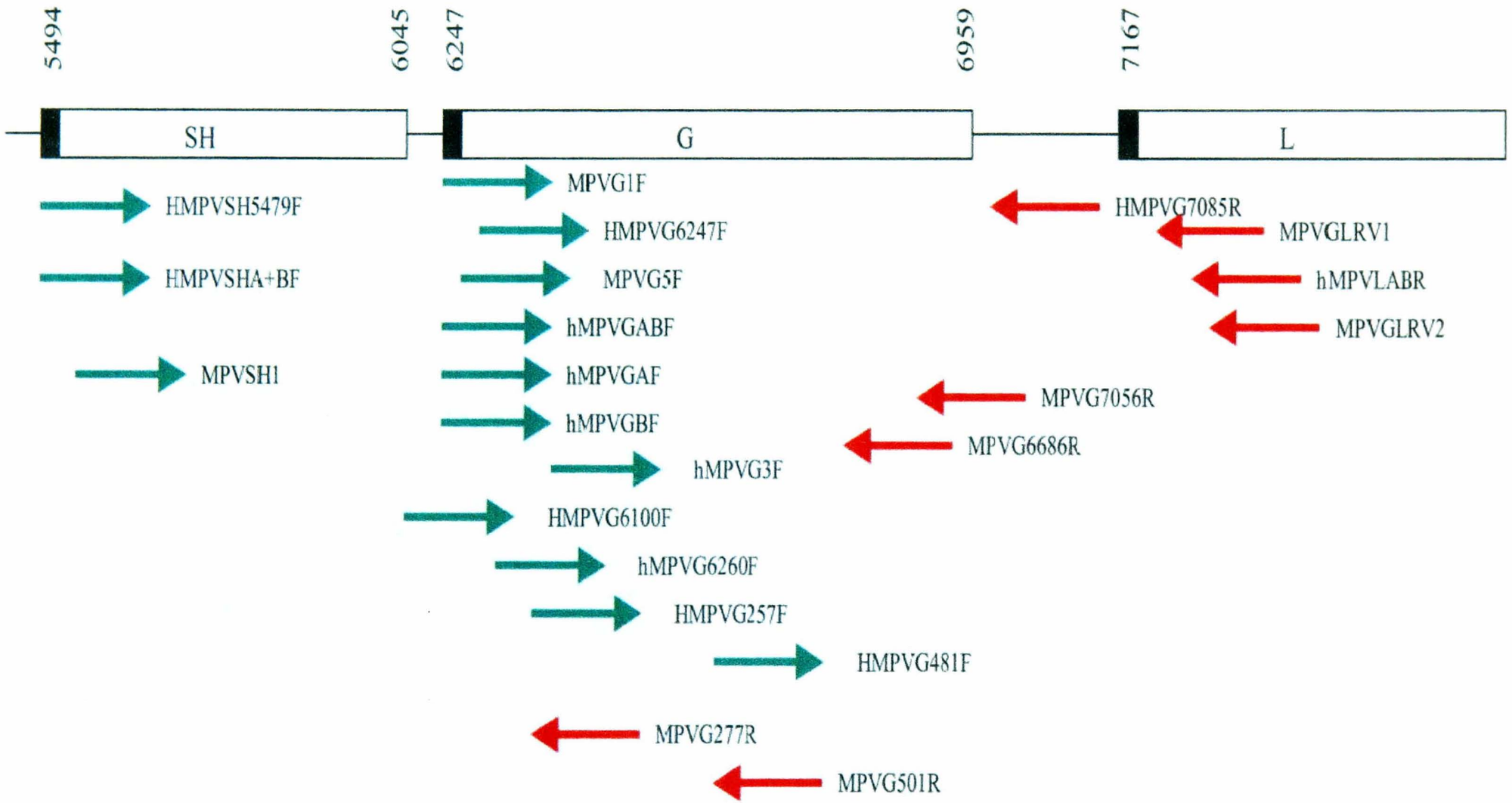


Figure 4.1: Schematic representation of hMPV G primers evaluated for used in the G ORF sequencing strategy. For primer sequences and nucleotide positions, see Table 3.1.

An example of the some of the nested PCRs, which yielded the correct sized products, are shown in Figure 4.2. Products were sequenced and confirmed to be the correct target.

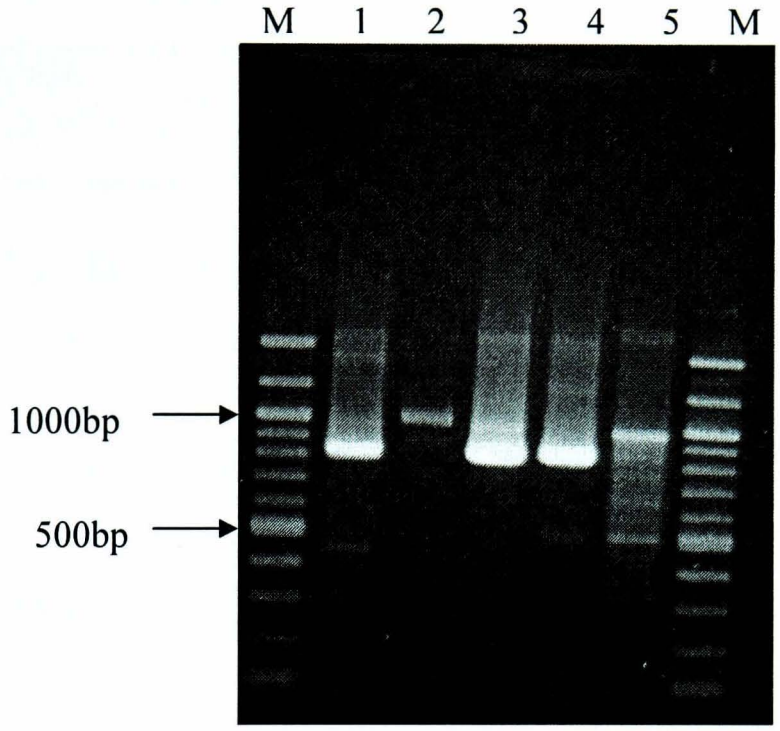


Figure 4.2: M is a mobility marker with base sizes indicated with arrows. Lane 1 contains the product amplified with primers hMPVG6100F and hMPVG6056R primary round, hMPVGABF and hMPVG7056R Secondary round. Lane 2 amplification product generated with primers hMPVG6100F and hMPVLABR primary round, hMPVGABF and hMPVG7085R. Lane 3 amplification product generated with primers hMPVG6100F and hMPVLABR primary round, hMPVGABF and hMPVG7056R. Lane 4 amplification product generated with primers hMPVG6100F and hMPVG7085R primary round, hMPVGABF and hMPVG7085R. Lane 5 Amplification product generated with primers hMPVG6100F and hMPVG7085R primary round, hMPVGABF and hMPVG7085R

The primer combinations, which were found to give the best amplification and sequencing results, are listed in Table 4.2.

Table 4.2: Selected hMPV G Primers used for the amplification of hMPV G ORF.

Primer	Sequence 5'-3'	Gene	Position *	Size
	Primary Amplification			
HMPVG6100F	TTT AAA AAT ATT TTG AAA AC	SH-G	6100	1104
HMPVLABR	AGA TAG ACA TTA ACA GTG GA	L	7204	
	Secondary Amplification			
HMPVGABF	GGG ACA AGT RGY YAT GGA RGT RAR AG	SH-G	6233	971
HMPVLABR	AGA TAG ACA TTA ACA GTG GA	L	7204	

Footnotes: (*) Primer positions according to reference strain NL00-1. F: Forward primer, R: reverse primer.

Screening of the Primer sets was carried out using a non-proof reading DNA Taq Polymerase. Therefore, a range of proof reading Taq polymerases were investigated to determine which would be the optimal to use in this PCR. PCRs were set up according to the manufacturer's instruction, and the primary and secondary products analysed on a gel (Figure 4.3).

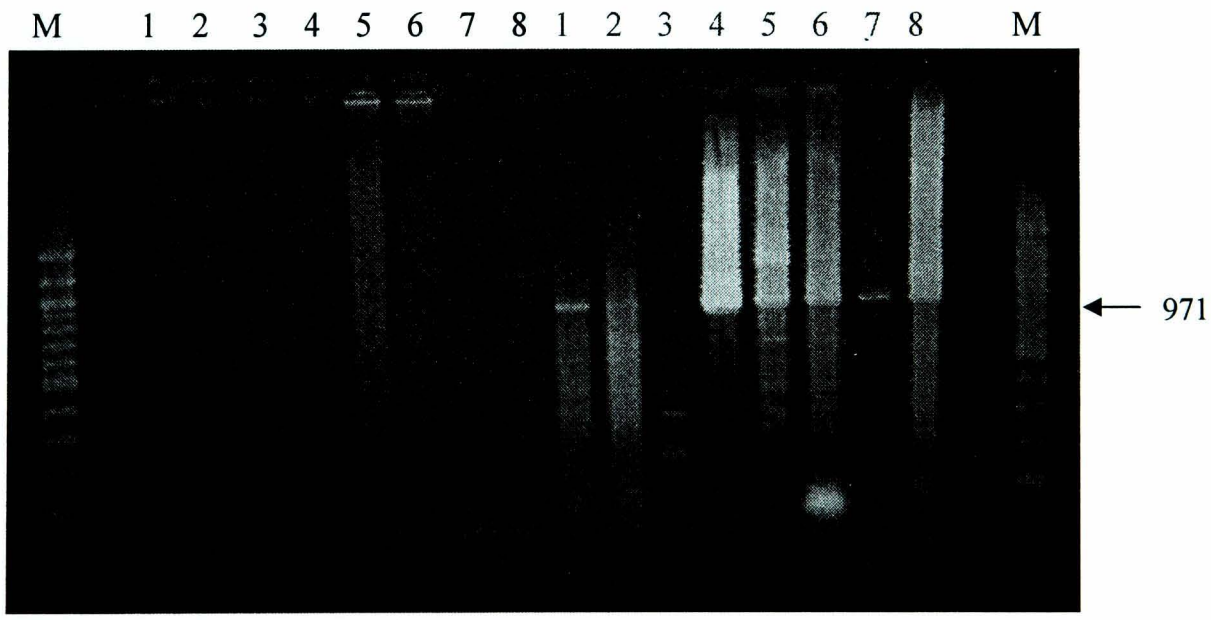


Figure 4.3: Determination of the optimal proof reading Taq polymerase. The lanes 1-8 in black are the primary round products, lanes 1-8 in Red are the secondary round products, amplified using (1) PFU Ultra, (2) PFU Turbo, (3) Expand, (4) Platinum Taq, (5) Platinum PFX, (6) AccuePrime PFX, (7) Platinum PCR SuperMix, (8) DNA Taq Polymerase.

Results shown that none of the Taq polymerises tested produced primary round products that could be visualised on a gel. However, the secondary round products show that there were considerable differences between the different proof reading Taq polymerases. All produced the correct sized product with the exception of Expand. Platinum Taq gave the strongest result and was therefore chosen for analysis of clinical strains.

4.3.2 Degenerate versus non degenerate primers

Up until this point the chosen primer set included a degenerate forward primer in the second round of the PCR. A degenerate primer was used to enable amplification from both subtypes of hMPV. However, the use of degenerate primers is often not as sensitive and results in the non-specific amplification of nucleic acid when compared to non-degenerate primers. Therefore, the performance of the degenerate primer was compared to A and B subtype specific non-degenerate primers at the same site (Table 4.3) against clinical material known to be either positive or negative for hMPV by the F gene RT-PCR described previously. Any bands generated were sequenced using the amplification primer set to confirm the specificity of the primers; the results of which are summarised in Table 4.4.

Table 4.3: Comparison of degenerate and non-degenerate primer sequences.

Primer	Sequence 5'-3'	Subtype
HMPVGABF	GGG ACA AGT RGY YAT GGA RGT RAR AG	A and B
HMPVGAF	GGG ACA AGT AGT TAT GGA GGT GAA AG	A
HMPVGBF	GGG ACA AGT GGC CAT GGA AGT AAG AG	B

Footnotes: Highlighted in Red are the degenerate bases in the hMPV G AB primer, highlighted in green are the bases selected for subtype specific detection. Degenerate bases R: A & G, Y: C & T.

Results show that of the 13-hMPV positive samples identified by hMPV F gene RT-PCR, 10 were also positive by one or more of the three hMPV G RT-PCRs tested. Overall the positivity rate using the hMPV G RT-PCR was 77%. The material used for this screening was the same cDNA material used for the initial F gene analysis.

The 3 samples in which the G gene was not amplified were highlighted in red. Bands that were slightly smaller than the expected size were generated in 2 out of these 3 samples. Sequence analysis revealed these bands were human chromosomal material.

Overall the use of subtype specific primer sets yielded a larger number of positive results than the degenerate primer set. Highlighted in green are examples of the subtype specific PCR outperforming the degenerate primer set.

Table 4.4: Summary of results comparing degenerate and subtype specific primers against hMPV positive and negative clinical samples.

		Primer Set				
Sample Number	**F GENE**	**AB**	**A**	**B**	**Sequence Result**	**Optimal Primer Set**
hMPV positive samples	P	P	P	WP	P	A
	P	P	P	WP	P	A
	P	WP	N	WP	P	B
	P	P	P	P	P	A or B or AB
	P	P	P	N	P	A
	P	P	P	N	P	A
	P	P	P	N	P	A
	P	N	N	WP	P	B
	P	WP	N	P	P	B
	P	WP	N	P	P	B
	P	N	N	N	N*	-
	P	N	N	N	N*	-
	P	N	N	N	N*	-
hMPV negative samples	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-
	N	N	N	N	-	-

Footnotes: (P)= positive (N)= negative (WP) = week positive. (*) Samples positive by the F gene PCR but not the G gene PCR. All 3 primer sets used the same primers for the first round PCR: HMPVG6100F, HMPVLABR the sequences of which are given in Table 4.2. For the second round the same reverse primer, HMPVLABR, was used in all three primer sets. Only the forward primer changed. AB: HMPVGABF, A: HMPVGAF, B: HMPVGBF, the sequences of which are given in Table 4.3.

The primary round PCR was therefore followed by each subtype specific PCR, as outlined in Figure 4.4.

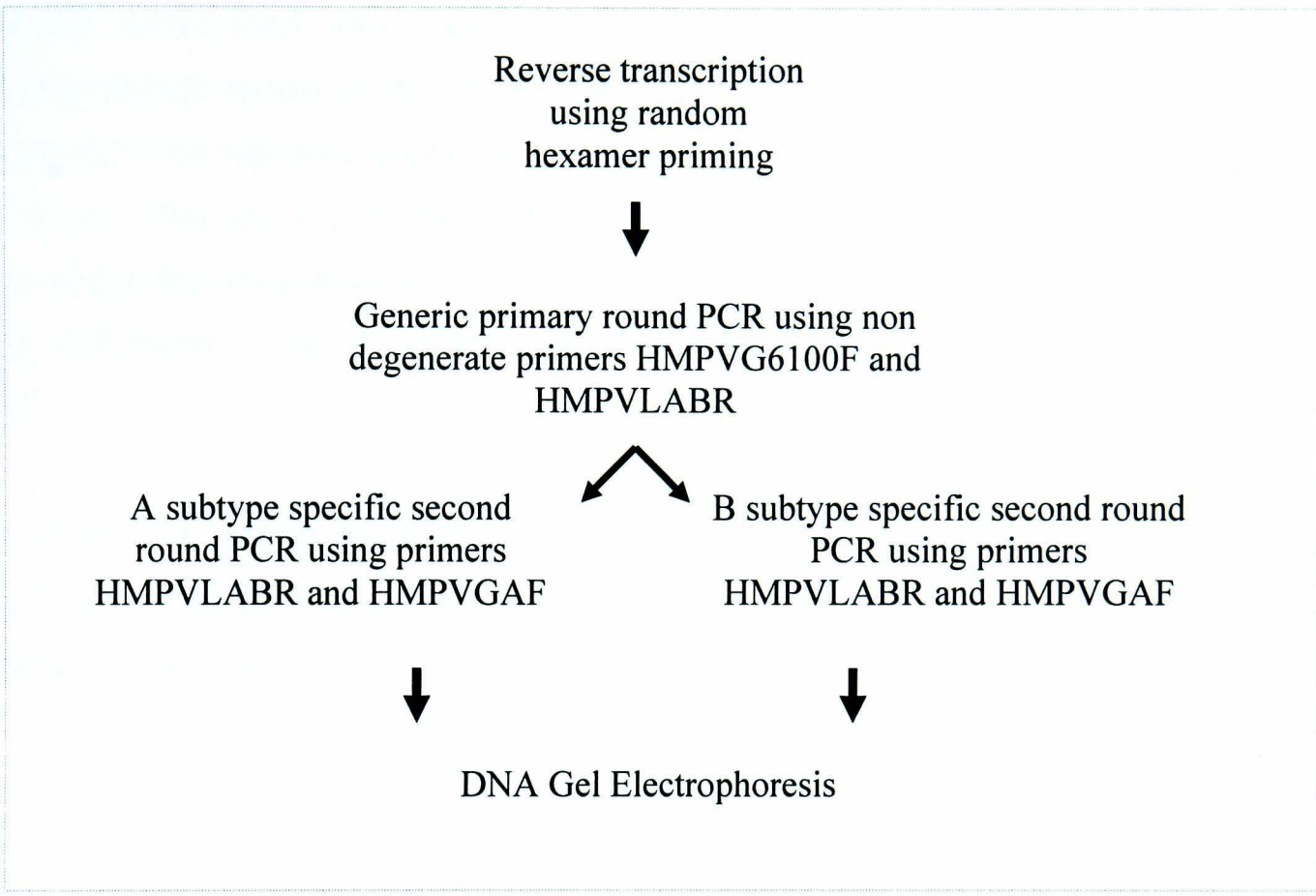


Figure 4.4: Amplification strategy for hMPV G ORF sequencing.

4.3.3 Cloning and design of sequencing primers

Due to the high level of variation expected to be seen in this gene it is difficult to determine before hand which internal primers to use in order to generate full length sequence on both strands of the DNA. Therefore to overcome this problem and to improve the quality of the sequence generated the purified PCR product was cloned in to the TOPO TA vector. This allowed a first round of sequencing to be performed using the M13 Forward and Reverse primers located in the vector, the data of which enabled choices to be made with respect to which internal primers should be used to generate further sequence data.

The TOPO TA cloning vector is a commercially available vector that is supplied in its linear form, with thymidine overhangs at the 3' ends. Coupled to topoisomerase I, which mediates the ligation.

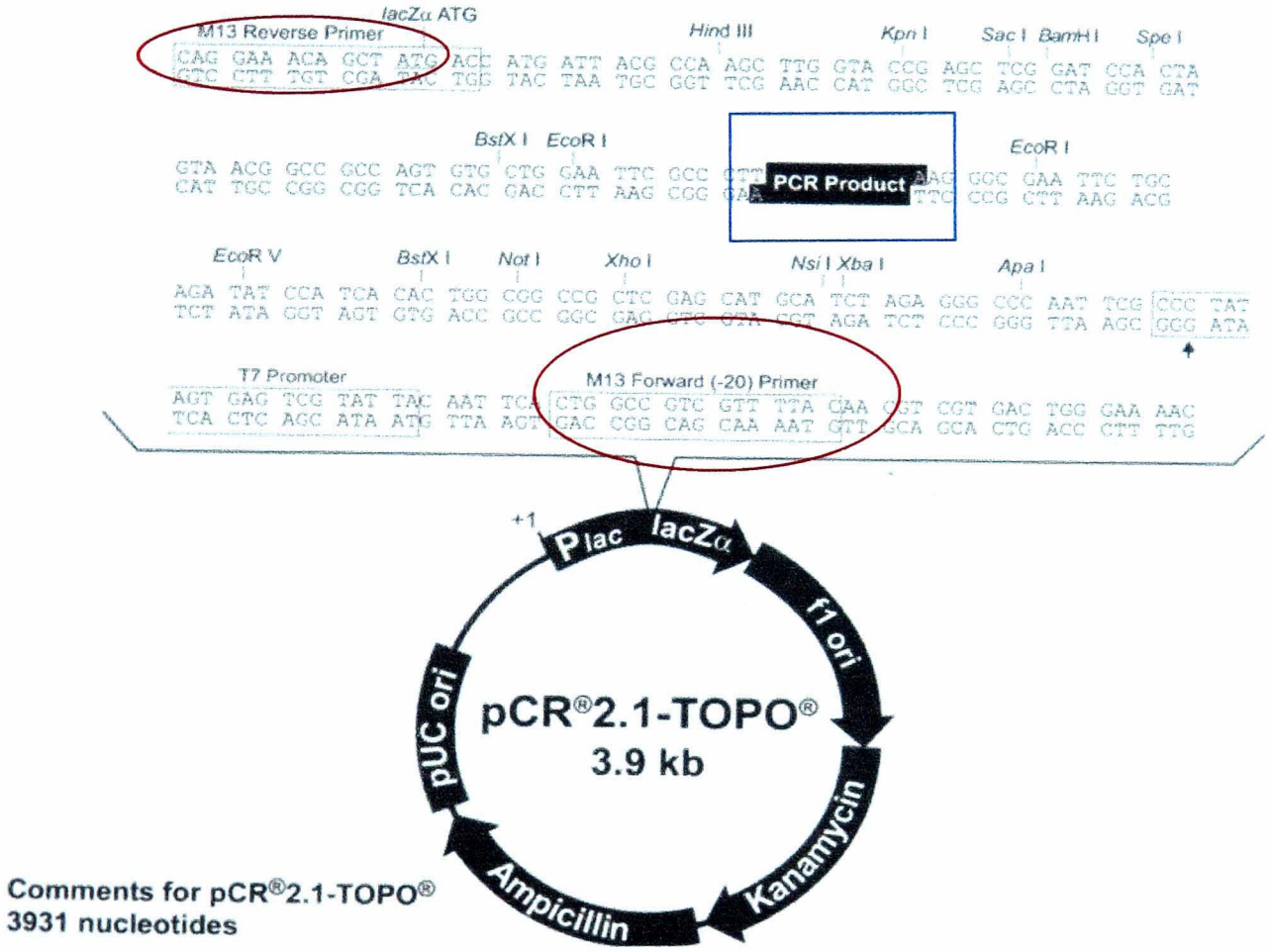


Figure 4.5: pCR 2.1 –TOPO Map shows the features of pCR2.1 TOPO and the sequence surrounding the cloning site which is highlighted by a blue box. The M13 forward and reverse primer positions are highlighted by red ovals. Taken from the Invitrogen TOPO TA cloning instruction manual Version P 051302 25-0184

This system allows the rapid cloning of purified PCR products that have the complementary adenosine overhangs at the 3' ends. Normally when a non-proof reading Taq-polymerase is used these 3' overhangs are added. However with a proof reading enzyme, they are removed. Therefore, the 3' adenosines were added to the ends of the PCR products, which were subsequently purified prior to cloning in to the TOPO vector, as described in materials and methods.

TOP 10 *E.coli* were transformed with the ligated vector, and plated on to ampicillin selective agar. Positive colonies were selected through blue/white screening, and grown up in culture. The plasmid DNA was purified and the presence of the insert confirmed by restriction digest (EcoR1). Sequencing was performed using the M13 forward and reverse primers, which prime either side of the cloning site in the TOPO vector. This provided a near to full-length sequence of the G gene. Based on the first round of sequencing data 6 additional primers were designed to generate overlapping sequences for both strands of the DNA, ensuring the full length of the gene was sequenced (See Chapter 2 Materials and Methods for primer sequences)

4.3.4 Analysis of quasispecies

Due to the nature of RNA genomes and the high degree of variation that occurs in the G gene especially, it is highly likely quasispecies exist within the same sample. Amplification by PCR is likely to amplify the major species present in the sample, or the one to which the primers bind most efficiently. Furthermore, the use of cloning may mean that the sequence obtained is not representative of the major species and may only represent a subset of the quasi species. Therefore to ascertain whether differences would occur between separate clones originating from the same sample, 2 clinical samples were randomly selected and the sequencing results from 5 separate clones from each sample were analysed for variation. Figure 4.6 shows the alignment results for 5 separate clones generated from each of the 2 samples.

Results show that the sequences obtained from the separate clones are identical with the exception of one amino acid difference at position 77 in only one out of the 5 sequences obtained from sample A, and 2 amino acid changes at positions 21 and 217 in one out of 5 of the sequences obtained from sample B.

The nucleotide mutation corresponding with the amino acid substitution at position 77 is sample A is an A to G transition at the 1st position in the codon. There are also 2 silent mutations observed in sequences obtained from sample A; an A to G nucleotide change at position 57 at the third base of the codon in one sequence and another A to G transition in the stop codon in the third base position creating an ATG stop codon in another sequence.

The nucleotide mutation corresponding with the amino acid substitution at position 21 in sample B is a C to T transition in the 1st position of the codon, and at position 217, a G to A transition also in the 1st base of the codon.

This indicates that quasispecies exist and that while there are a few amino acid changes that may be missed on the whole the sequence data from one clone is likely to be a good representation of the majority species in the sample.

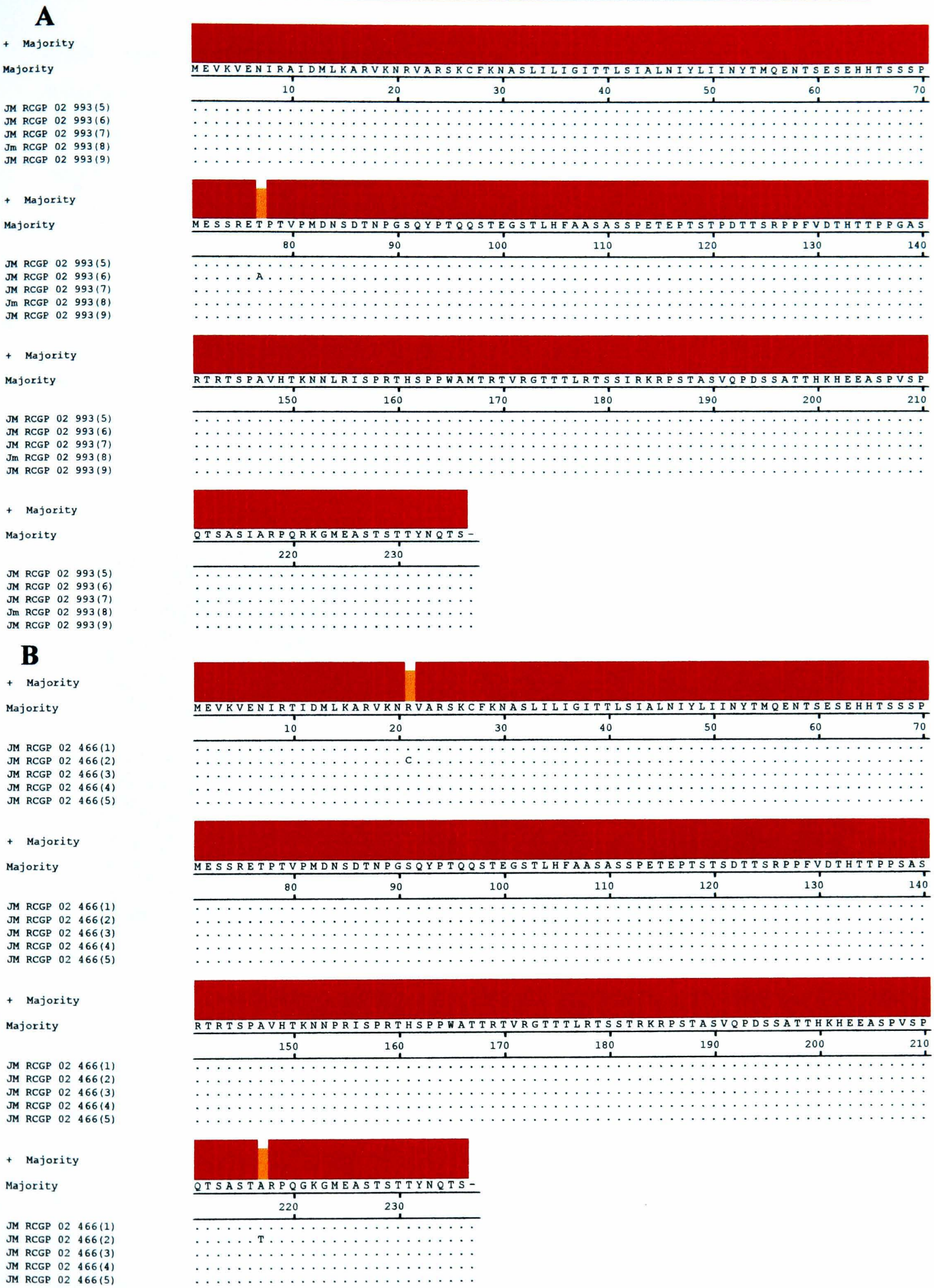


Figure 4.6: Sequence alignments of the full-length attachment glycoprotein from samples A and B, where 5 separate clones were generated from each sample. Red represents areas of identical amino acid. Orange where amino acid differences are present.

4.3.5 Analysis of clinical samples

Nose and throat swabs from the community acquired and hospitalised paediatric studies identified as being positive for hMPV by the F gene RT-PCR were analysed with the hMPV G PCR excluding samples in which dual infections were identified. Wherever possible G was amplified from the same cDNA from which the initial F gene analysis was performed. However, for many of the samples there was insufficient cDNA available and therefore the original sample was re-extracted. An example of the hMPV G-A and G-B subtype PCR against clinical samples is shown in Figure 4.7. Results show that that there is often other non-specific bands generated, which appears to be greater with the hMPV G-subtype B primer set, compared to that of subtype A.

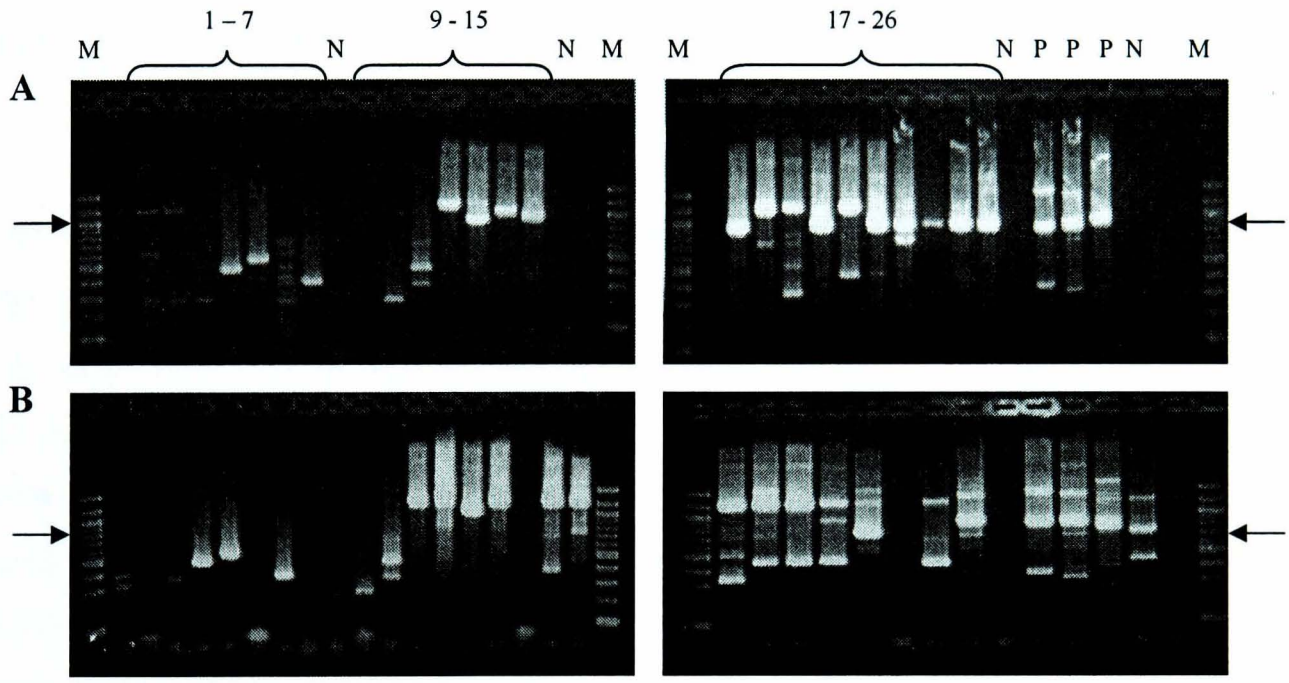


Figure 4.7: Example of the hMPV G subtype A and B PCRS on clinical samples. M is the mobility marker, 1-7, 9-15, and 17-26 are all hMPV positive clinical samples. N indicates negative controls, P represents positive control samples. The Arrow represents the expected size of the product.

Table 4.5 summarises the use of the sequencing strategy for the different study groups. Overall, of the 135 samples that were positive for hMPV, 103 were hMPV only infections (NB: Rhionviruses and coronaviruses were not investigated in the community study). All 103 samples were analysed using the hMPV A and B G gene PCR for sequencing. Of these approximately 50% yielded bands of the correct or approximate size, and were cloned in to the TOPO vector and sequenced. Of these 30 were found to be the correct target.

Table 4.5: Summary of the hMPV G sequences obtain from this work.

Season	Study	Total hMPV	hMPV only (%)	hMPV Sequence (%)	hMPV A (%)	hMPV B (%)
01-02	Community	14	14 (100)	7 (50)	7 (100)	0 (0)
02-03		19	11 (57.9)	1 (9)	0 (0)	1(100)
01-02	Paediatric	27	26 (96.3)	11 (42.3)	11 (100)	0 (0)
02-03		40	24 (58.5)	10 (42)	10 (100)	0 (0)
Total		135	103 (76)	29 (30)	28 (96)	1(4)

Footnotes: Each study group is broken down in to the two years in which samples were analysed for hMPV G sequence. Column 3 shows the number of hMPV positive samples detected in that group. Column 4 shows the numbers which were positive only for hMPV. Column 5 the number of full length G gene sequences obtained for that group. Column 5 and 6 the number of A or B subtype sequences obtained.

The specificity of the primers was not evaluated in this study, however, Blast analysis of the sequence generated from some of the additional bands revealed that segments of the sequences shared homology with Homo sapien chromosome 10 (unspecified locus), and Human aldo-keto reductase (Family of enzymes that catalyse the NADPH-dependent reduction of a number of compounds) with E values of $7e^{-167}$ and $5e^{-51}$ respectively. In a crude nucleotide alignment performed using the DNA Star software megalign there was between 29 and 33% percent identity between the nucleotide sequences generated from these bands and the hMPV reference strains used in the phylogenetic analysis presented in this thesis.

4.4 Phylogenetic Analysis

Amino acid alignments of the different data sets were created using Clustal X, and applied to CODON Align with the corresponding nucleotide sequences in order to create in-frame nucleotide alignments. The in-frame nucleotide alignments were subsequently used for the generation of maximum likelihood trees using the program PAUP (4.10b). The criterion used for creating the trees was determined using the MODELTEST 3.7 (Posanda and Crandall 1998) application within PAUP (Table 4.6). This application compares different models of evolution and determines which model and criterion best fit the data set under investigation. Bootstrapping of maximum likelihood trees was impractical due to the large amount of computing time required. Maximum likelihood trees were therefore compared with bootstrapped neighbour joining trees. Where trees were congruent bootstraps are shown on the maximum likelihood trees.

The maximum parsimony methods were not used in this work as it best suits closely related sequences.

In-frame nucleotide alignments were used to calculate Transition: Transversion Ratios using the program PUZZLE, and Non-synonymous: Synonymous Ratios using the program MEGA 3.1.

Figures 4.8 and 4.9 show the phylogenetic relationships of all the available hMPV full length G ORF sequences in neighbour joining trees. Figure 4.8 was created in DNASTAR and is based on the amino acid alignment presented in Figure 4.15. Figure 4.9 is based on the in frame nucleotide alignment created from figure 4.15 in codon align. All in-frame nucleotide alignments can be found in Appendix 2. Unfortunately due to time constraints bootstrap values were not generated for these two trees. Both figures however show clearly the division of strains in to 2 major lineages A and B, which are both further subdivided in to 2 sub-lineages A1, A2 and B1, B2.

Sequences generated in this study are highlighted in blue (strains from hospitalised children) or green (strains from community acquired ILI) .It is clear that all but one of the strains from this study belong to the A lineage. It is also clear that the strains from this work and that of others are all very closely related to one another, and the short branch

lengths indicate that very little accumulation of amino acid changes has occurred. There is therefore no difference between strains circulation in the community or those identified in hospitalised children

The strains circled in yellow are outliers of the A1 sub-lineage that show unusual mutations which alter drastically the amino acid sequence of the down stream protein, and are highlighted in yellow in figure 4.15. These may be naturally occurring mutations or may have arisen as a result of only having sequenced one clone. Where these sequences have been treated separately in further analysis they have been referred to as the A1 outliers and have been discussed in detail later in this chapter.

Figures 4.10 and 4.11 are maximum likelihood trees showing the close phylogenetic relationship of strains sequenced in this study from the community or hospitalised children respectively. Again it is apparent that very little antigenic drift is occurring in the G protein of these isolates.

Figure 4.12 is a maximum likelihood tree of all the strains sequenced in this study, and is highlighted according to the year of circulation. This tree again indicates the same strains circulating during year 1 (2001-2002) of the community and hospitalised children's studies, phylogeneticaly very closely related to those circulating in year 2 (2002-2003) of the study.

Figures 4.13 and 4.14 show again in maximum likelihood trees this time the phylogenetic relationships of A or B lineages respectively, and confirm that findings of the neighbour joining trees shown in figures 4.8 and 4.9.

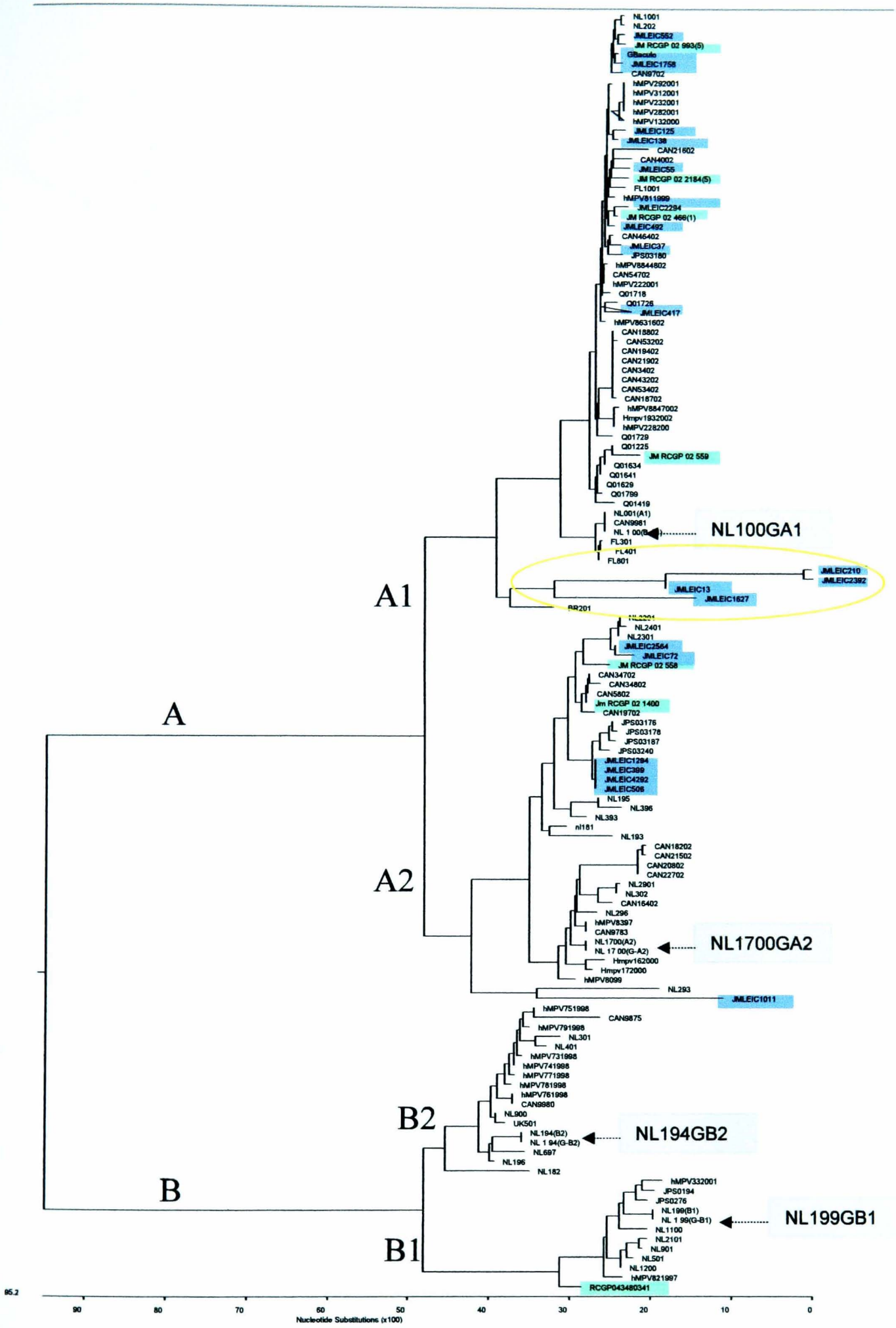


Figure 4.8: Neighbour joining tree of all available full length G sequences based on the amino acid alignment presented in Figure 4.15. Reference Strains are highlighted in grey; Sequences from this work are highlighted in (green) Hospitalised children, or (Blue) Community Acquired. Tree is mid-point rooted for clarity. Created in DNA STAR.

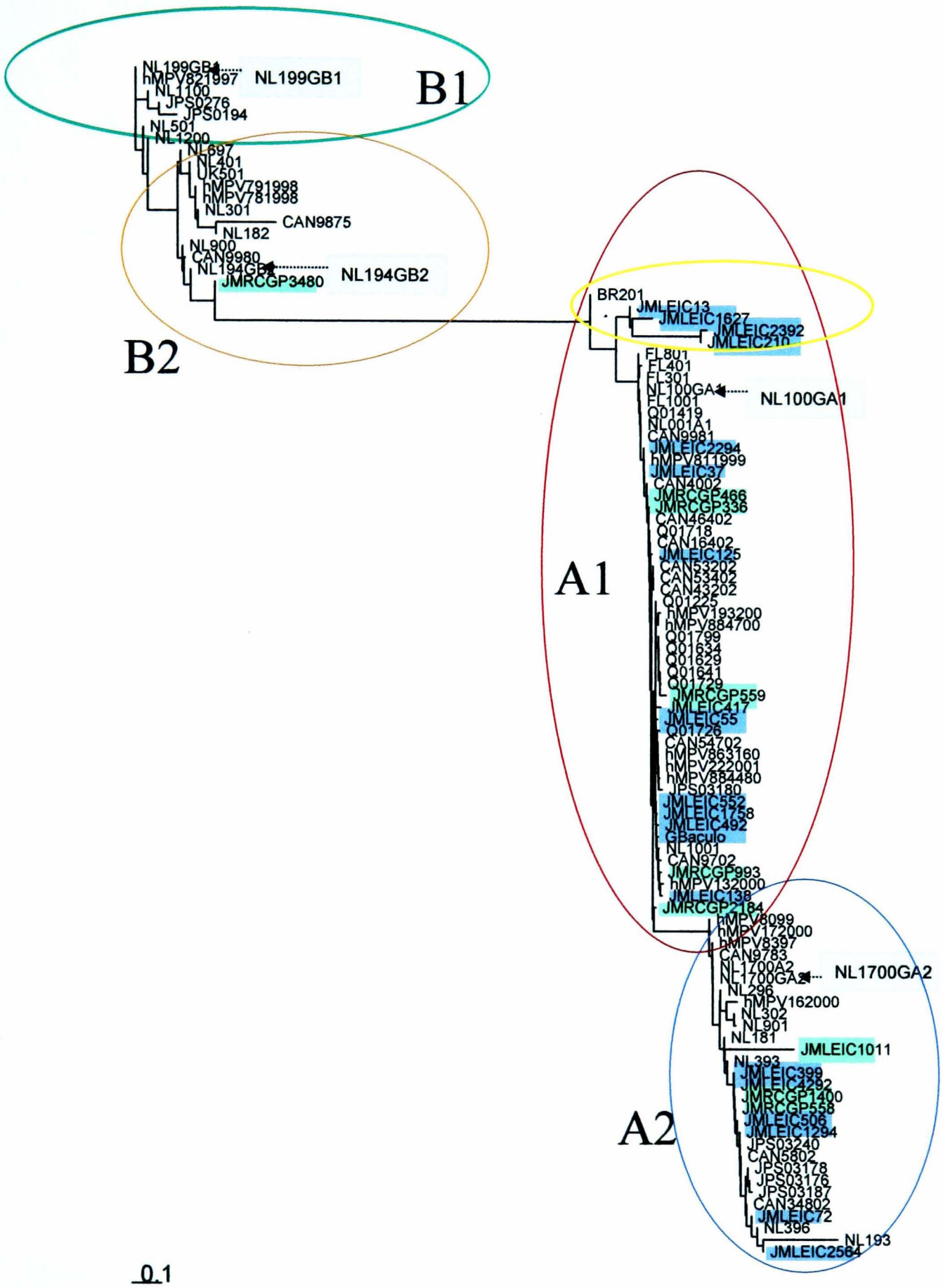


Figure 4.9: Neighbour joining tree of all available full-length G sequences based on in frame nucleotide alignment of the amino acid alignment in Figure 4.15. Reference Strains are highlighted in grey; Sequences from this work are highlighted in (Blue) Hospitalised children, and (Green) Community Acquired. Consensus tree based on 500 replicates

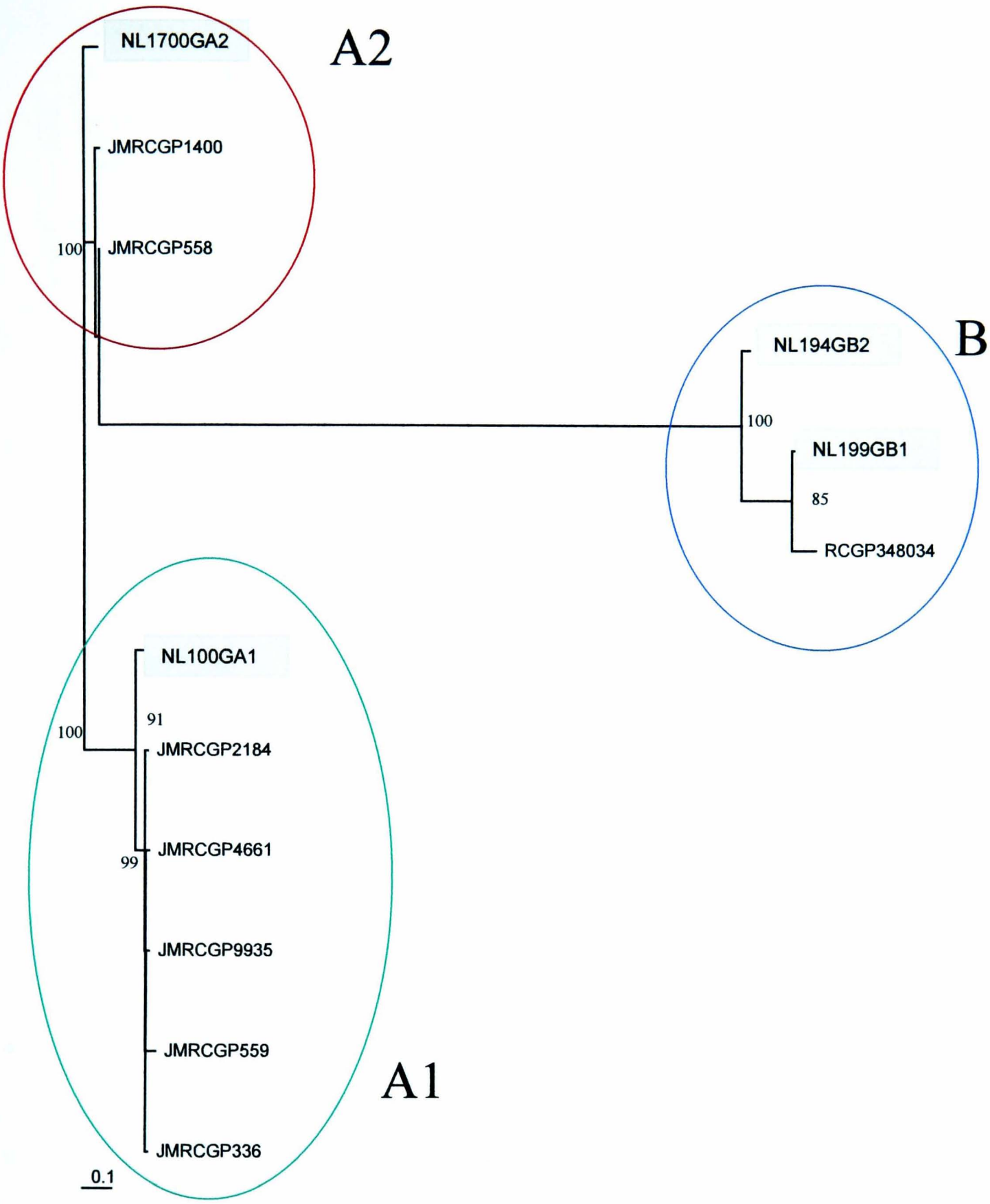


Figure 4.10: Maximum likelihood tree of samples acquired from the community. The tree is based on in frame nucleotide alignment generated in CODON Align from the corresponding amino acid alignment shown in appendix II. Reference strains are highlighted in grey. Boot strap values are based on congruent neighbour joining trees of 1000 bootstraps.

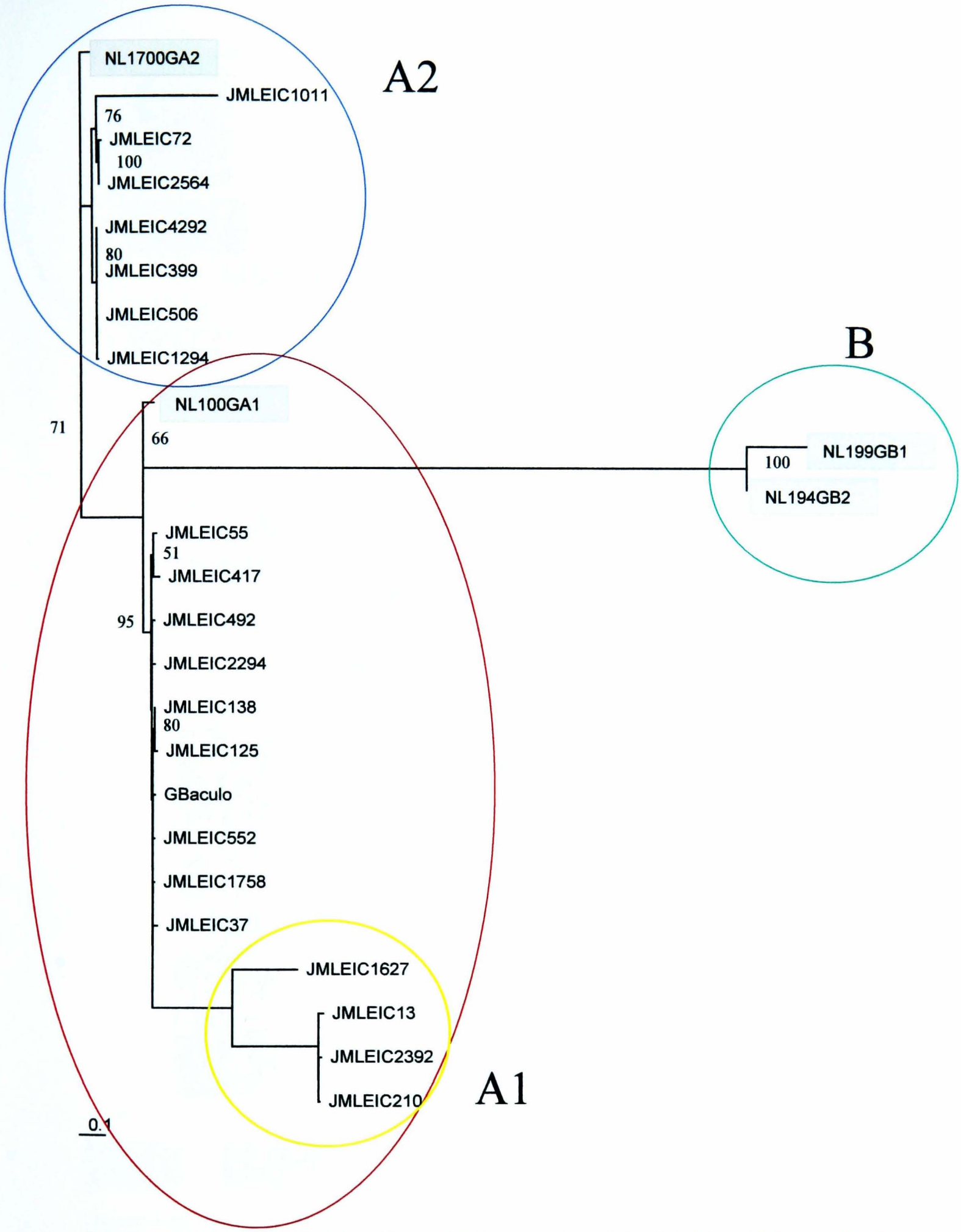


Figure 4.11: Maximum likelihood tree of strains from hospitalised children. Reference Strains are highlighted in grey. The tree is based on in frame nucleotide alignment generated in CODON Align from the corresponding amino acid alignment shown in appendix II. Boot strap values are based on congruent neighbour joining trees of 1000 bootstraps.

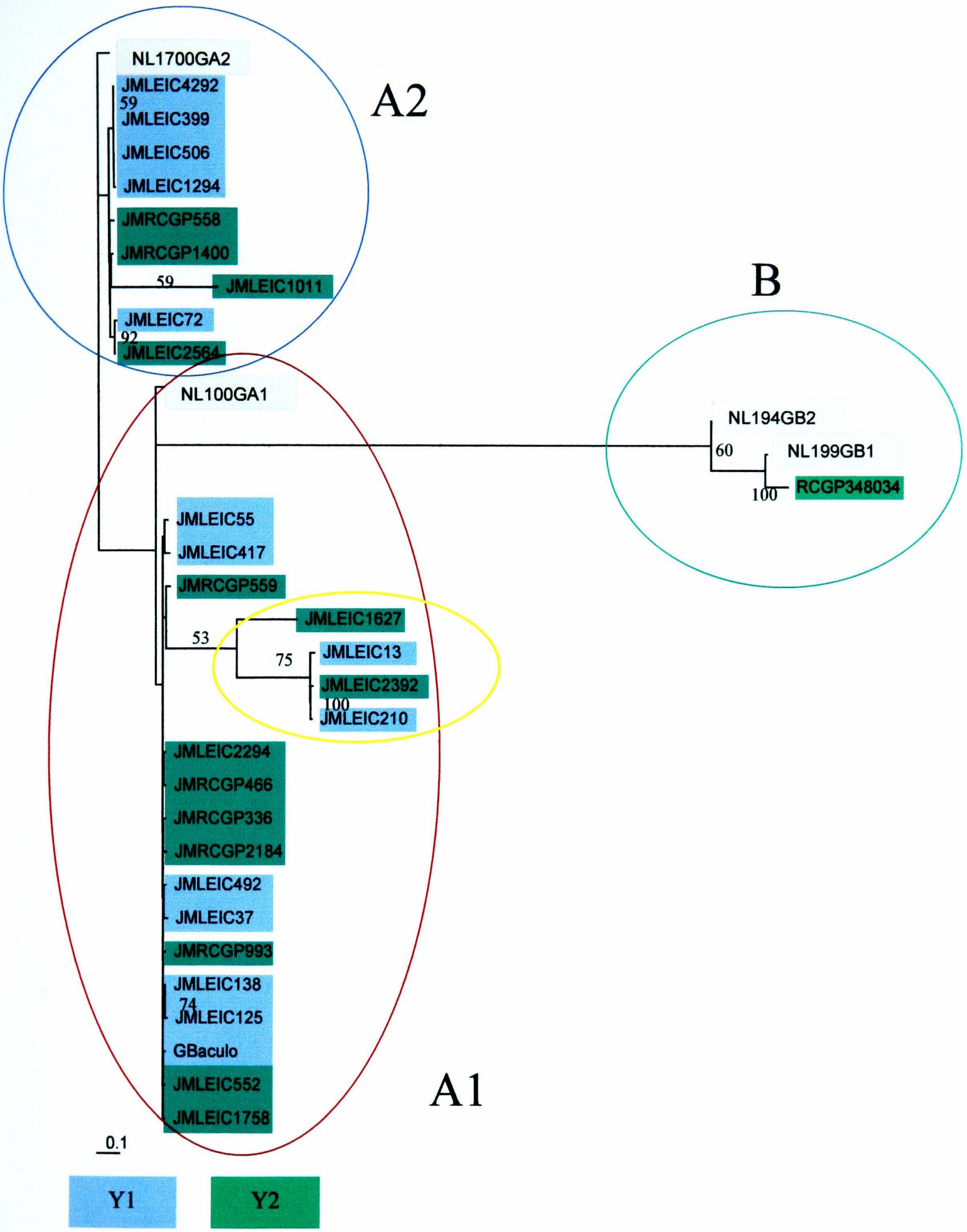


Figure 4.12: Maximum likelihood tree of hMPV G full-length sequences generated in this study and colour coded according to the study year. Y1 = 2001-2002, Y2 = 2002-2003. Reference Strains are highlighted in grey. The tree is based on in frame nucleotide alignment generated in CODON Align from the corresponding amino acid alignment shown in appendix II. Boot strap values are based on congruent neighbour joining trees of 1000 bootstraps.

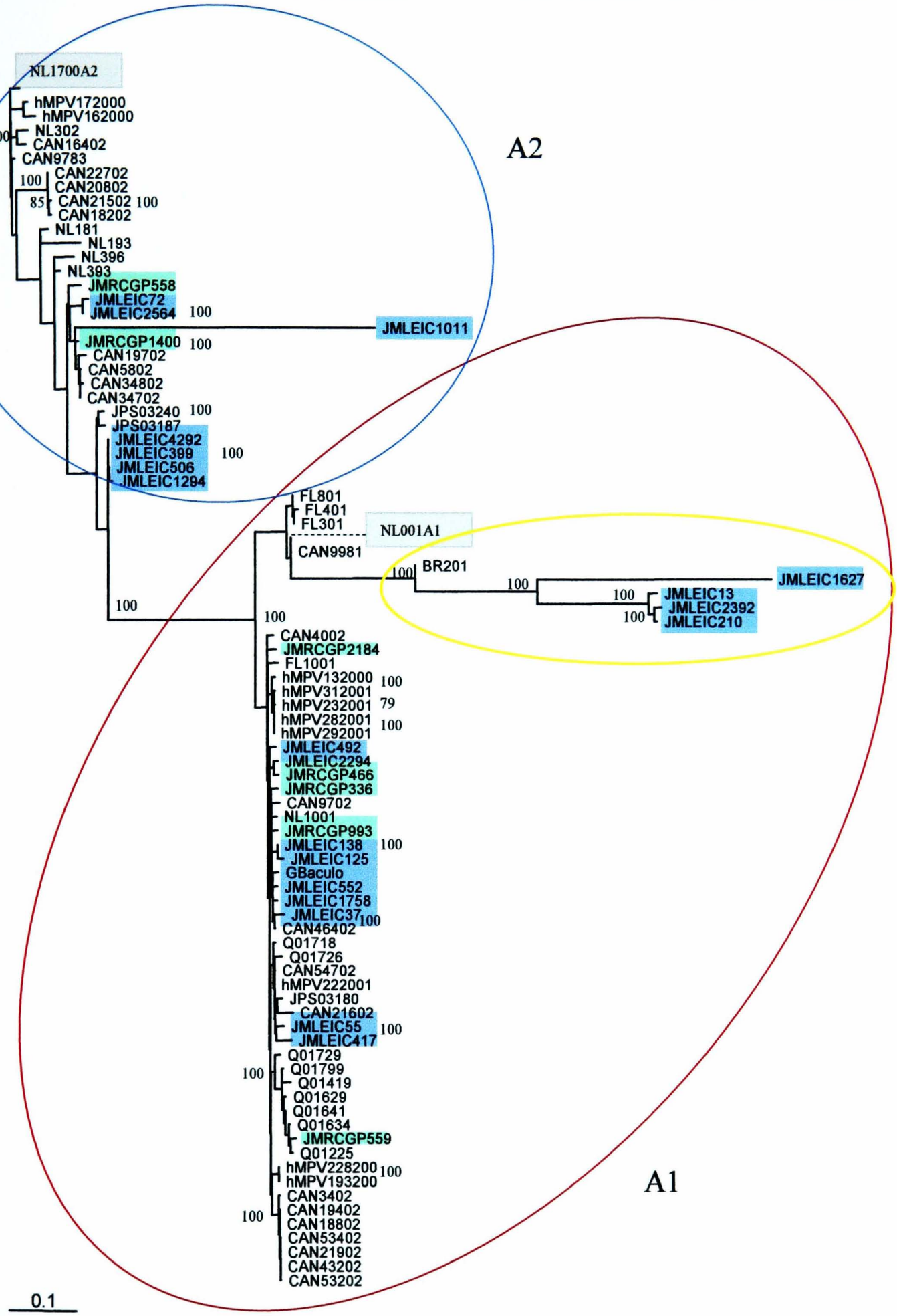


Figure 4.13: Maximum likelihood tree of all available Full-length G Subtype A sequences. Reference Strains are highlighted in grey; sequences from this work are highlighted in blue (Hospitalised children), green (Community Acquired). The tree is based on in frame nucleotide alignment generated in CODON Align from the corresponding amino acid alignment shown in appendix II.. Boot strap values are based on congruent neighbour joining trees of 1000 bootstraps.

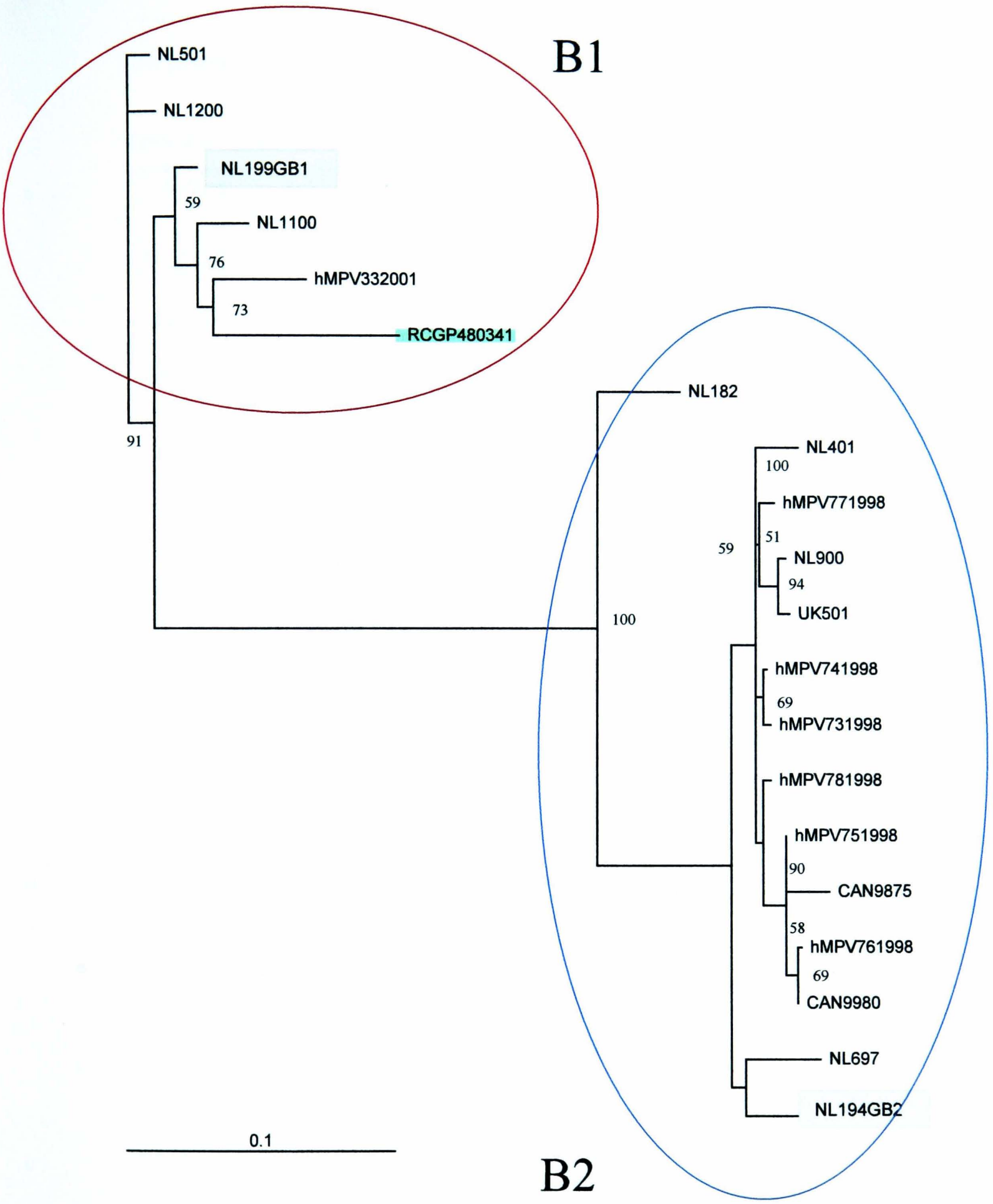


Figure 4.14: Maximum likelihood tree of all available Full-length G subtype B sequences. Reference strains are highlighted in grey; sequences from this work are highlighted in blue (Hospitalised children), green (Community Acquired). The tree is based on in frame nucleotide alignment generated in CODON Align from the corresponding amino acid alignment shown in appendix II. Boot strap values are based on congruent neighbour joining trees of 1000 bootstraps.

The parameters used for the above phylogenetic analysis are displayed below.

Table 4.6: Nucleotide substitution Rate matrix: Parameters used for Phylogenetic analysis.

Group	A-C	A-G	A-T	C-G	C-T	G-T	Invariable sites	γ Distribution
Community	1.00	7.25	1.00	1.00	18.14	1.00	0.19	5.76
Hospital	0.73	2.80	0.41	0.38	7.23	1.00	1.22	1.10
This study All	0.79	2.90	0.51	0.34	8.22	1.00	0.12	1.06
All A	1.00	3.51	0.52	0.52	9.64	1.00	0.17	1.51
All B	1.00	7.13	1.00	1.00	19.77	1.00	0.00	1.04

Footnotes: values calculated using the Model Block Test application in the program PAUP. Columns 2 through to 7 show the relative proportions of change between the specified nucleotides. The table also shows the Proportion of Invariable sites and the Gamma distribution shape parameter.

Ts/Tv Ratios

Table 4.7: Ts/Tv Ratios for the different datasets analysed.

Group (No of Seq)	Ts/Tv Ratio	Pyrimidine Ts/ Purine Ts	% Constant	% A	% C	% G	% T
Community (12)	2.93	0.62	32.8	39.3	30.0	16.9	13.7
Hospital (25)	2.49	0.65	29.0	38.7	30.4	16.9	14.0
RVU All (33)	2.13	0.63	28.2	38.7	30.3	17.0	14.0
All A (86)	2.23	0.66	36.1	38.5	30.5	17.0	14.0
All B (20)	4.69	0.55	53.9	41.7	28.6	16.5	13.2
All A1 (58)	2.61	0.63	44.3	31.8	30.5	17.6	13.8
All A2 (29)	2.42	0.72	35.3	39.3	30.6	15.7	14.4
All B1 (6)	3.42	0.46	75.6	42.7	29.3	16.9	11.2
All B2 (14)	7.22	0.59	76.0	41.4	28.4	16.3	14.0

Footnotes: Calculated in the program PUZZLE using the model of substitution: HKY (Hasegawa et al. 1985). (% constant) % of nucleotides constant in the multiple alignments. (% A/C/G/T) % of corresponding nucleotide bases in consensus sequence.

The transition: transversion ratios calculated in Puzzle indicates that as expected there are generally more transitions than transversions occurring. There is a marked difference in the Ts/Tv ratios between A and B subtypes, with a greater number of transversion occurring in the A dataset suggesting that there is greater selective pressure on A subtypes than B, although this may be skewed by the larger number of available A sequences. However, analysis between the B1 and B2 viruses show that transversions occurred far less frequently in B2 viruses than B1, despite the larger number of sequences under investigation. There is very little difference in the Ts/Tv ratios between viruses sequenced in this study that were circulating in the community and those in-hospitalised children.

The Pyrimidine Ts/ Purine Ts ratios indicate that the majority of transitions are occurring between Purine bases A and G for all data sets. The results also indicate that the G gene is adenine rich, with adenosine residues occurring approximately 40% of the time.

4.4.1 Synonymous: Non-synonymous Ratios

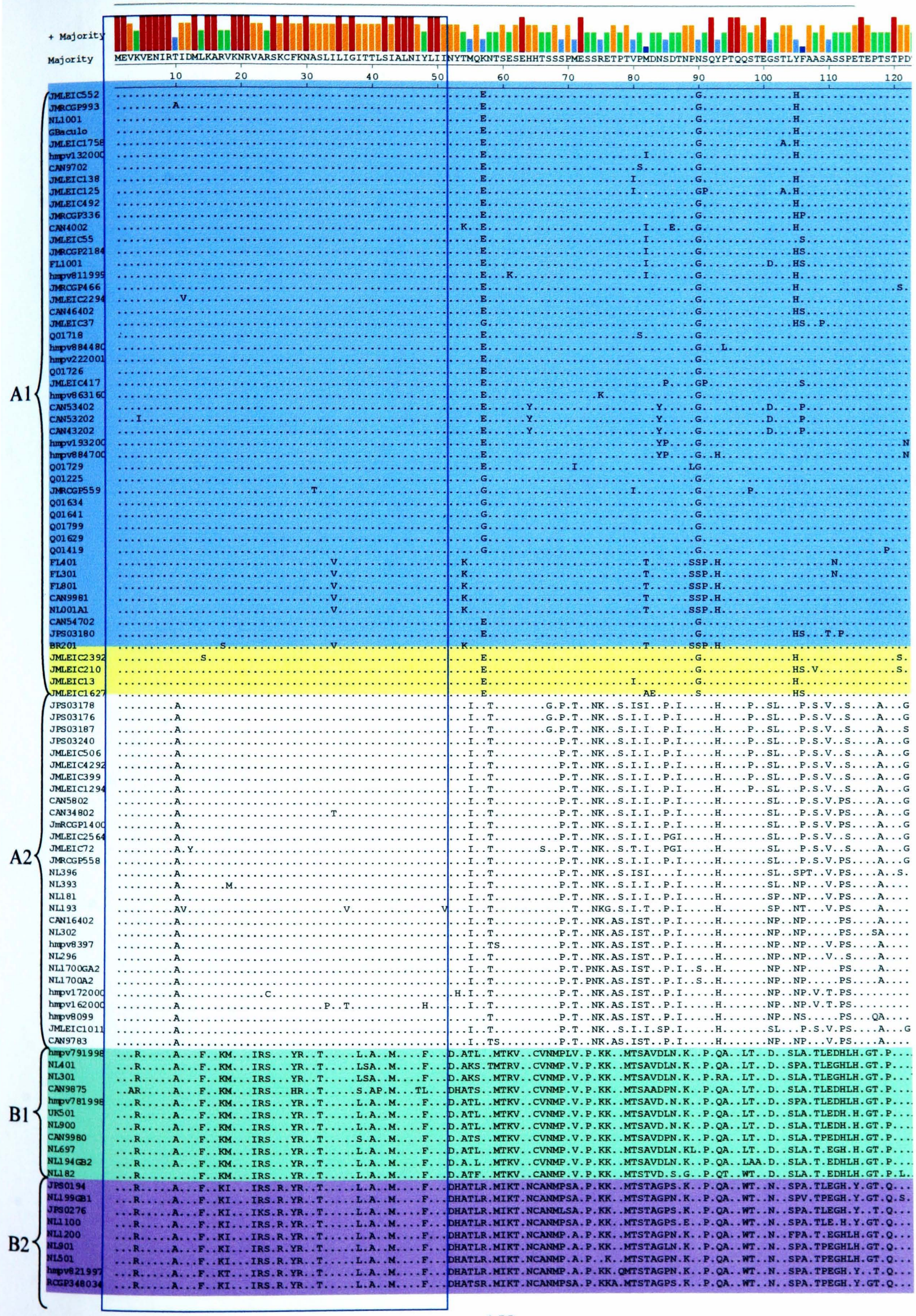
Table 4.8: Non synonymous: Synonymous ratios of the different data sets.

Group (No of Seq)	dN	dS	dN/dS	No of Syn Sites	No of non Syn sites
Community (12)	0.22	0.32	0.68	143	444
(SE)	(0.01)	(0.02)		(3.76)	(3.68)
Hospital (25)	0.17	0.24	0.71	137	426
(SE)	(0.01)	(0.01)		(3.71)	(3.96)
RVU All (33)	0.16	0.24	0.67	138	425
(SE)	(0.01)	(0.01)		(3.85)	(3.51)
All A (86)	0.10	0.15	0.67	142	436
(SE)	(0.00)	(0.01)		(4.04)	(3.97)
All B (20)	0.07	0.14	0.52	141	446
(SE)	(0.00)	(0.01)		(4.70)	(4.52)
All A1 (58)	0.05	0.08	0.61	142	436
(SE)	(0.00)	(0.00)		(4.20)	(4.31)
All A2 (29)	0.08	0.14	0.60	149	462
(SE)	(0.00)	(0.01)		(4.00)	(4.28)
All B1 (6)	0.04	0.09	0.43	141	461
(SE)	(0.00)	(0.01)		(4.51)	(4.49)
All` B2 (14)	0.03	0.06	0.472	157	499
(SE)	(0.00)	(0.00)		(4.95)	(4.75)

Footnotes: Calculated using the program Mega 3.1 using the Nei-Gojobori method (p-distance) with 500 replicates. (dN) non-synonymous, (dS) synonymous, (dN/dS) nonsynonymous: synonymous ratio.

The effects of nucleotide substitutions are classified as synonymous – silent mutations that do not alter the amino acid, and non- synonymous – mutations that do alter the amino acid. Table 7 shows the non-synonymous: synonymous ratios for the different data sets. In all

data sets the number of non- synonymous sites is approximately 3 times that of synonymous sites. However, as would be expected, there is a greater number of synonymous changes occurring, which is most apparent in Subtype B viruses. The significance of this compared with A viruses has not been established, but does suggest there may be greater selective pressure on subtype A viruses.


+ Majority
Majority
MEVKVENIRTIDMLKARVKNRVARSKCFKNASLILIGITTLSIALNIYLIINYTMQKNTSESEHHTSSSPMESSRETPTVPMDNSDTNPNSQYPTQQSTEGSTLYFAASASSPETEPTSTPD
10 20 30 40 50 60 70 80 90 100 110 120
A1
A2
B1
B2
152

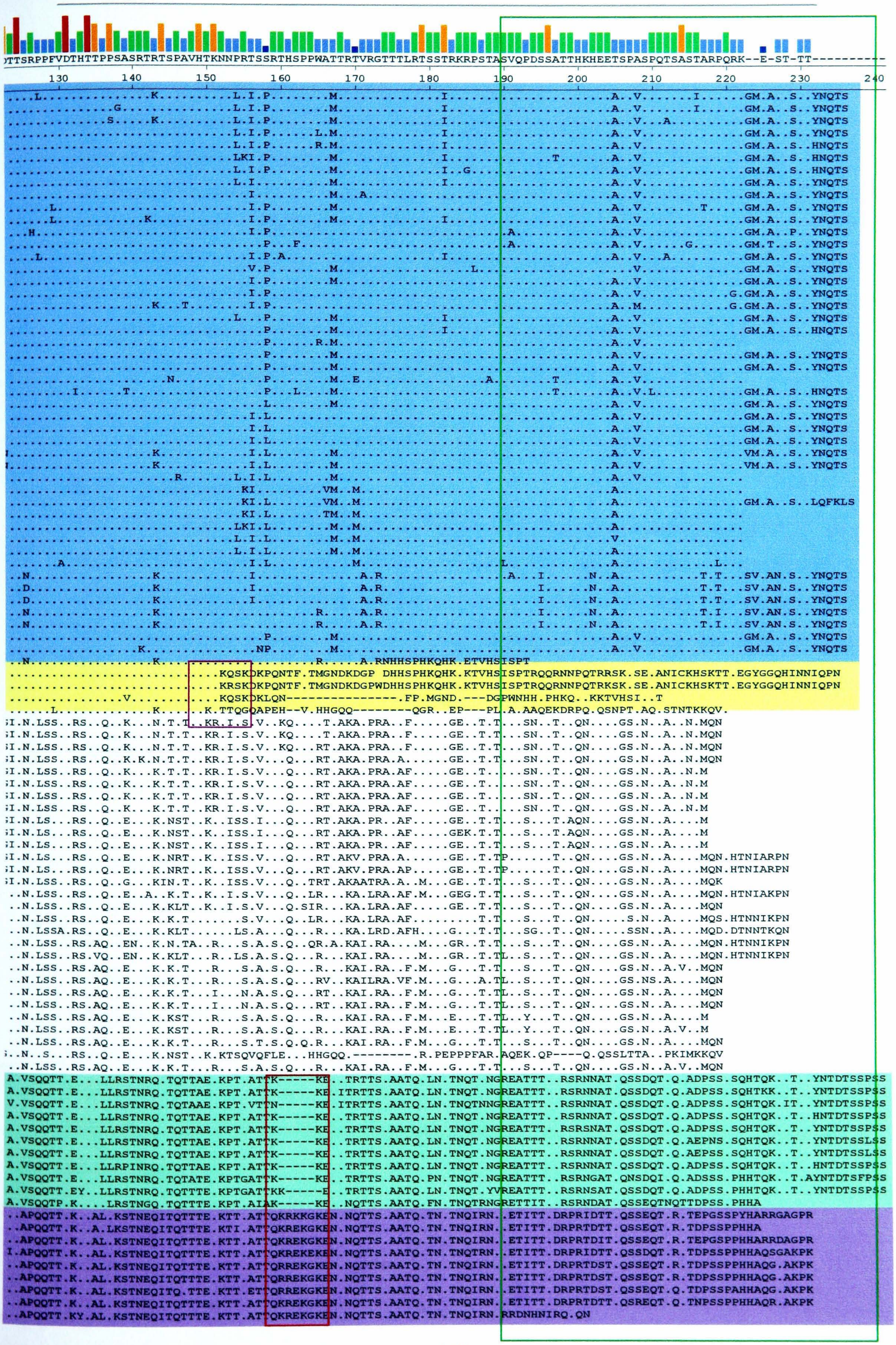

TTSRPPFVDTHTTPPSASRTRTSPAVHTKNNPRTSSRTHSPPWATTRTVRGTTTLRTSSTRKRPSTASVQPDSSATTHKHEETSPASPQTSASTARPQRK--E-ST-TT----------
130
140
150
160
170
180
190
200
210
220
230
240

Figure 4.15: Amino acid alignment of available sequences for hMPV G. The alignment was performed in Clustal X and imported in to DNASTAR Megalign for display purposes. The alignment has been filtered to hide amino acids that match the consensus (Majority are A1 Subtypes). Sequences highlighted in blue belong to the A1 subgroup. Sequences in yellow are the A1 outliers. Sequences in White A2, Green B2, and Purple B1. Across the top of the alignment the coloured histogram shows the consensus strength, with the most conserved positions in Red and the least conserved in dark blue. Amino acid deletion; (.) amino acids which match the consensus sequence of the alignment. Boxes highlight areas of interest. Blue box: Cytoplasmic and transmembrane regions of the protein. Green Box: Variable lengths at the carboxyl terminal. Red box: multiple insertion sites. Purple boxes: mutations resulting in change of ORF.

4.4.2 Analysis of variation

The amino acid alignment shown in Figure 4.15 highlights visually the extent of the variation that occurs in the attachment glycoprotein between, and within different subtypes of the virus. Highlighted in the coloured boxes are regions of interest. The blue box at the N-terminus of the protein shows the highly conserved cytoplasmic and transmembrane region. The green box at the carboxyl terminus highlights the variation in the lengths of the different proteins, and in red (residues 160 to 170), a possible insertion or deletion site containing multi basic residues in the B1/B2 subtype viruses. Purple boxes highlight potential changes in the ORF.

The percentage nucleotide identity of the alignment is displayed in Table 4.9 and the corresponding amino acid homologies in table 4.10. The results show that A and B subtypes share between 28-38% amino acid identity and 53-61 % nucleotide similarity. Within A subtypes there is between 50-100% and 78-100% amino acid and nucleotide similarity respectively. Finally within B subtypes there are 65-99 %, and 65-99% amino acid and nucleotide identity respectively. The A1 outliers are treated separately as to avoid potentially skewing results. What is also noticeable is that the amino acid variation is greater than that of the nucleotide, suggesting the nucleotide substitutions are advantageous for the virus.

Table 4.9: Percentage nucleotide identity within and between the different subtypes of the hMPV G protein.

Group	A1	A2	A1 Outliers	B1	B2
A1	90.5-100	-	-	-	-
A2	78.3-100	81.7-100	-	-	-
A1Outliers	92.4-99.3	78.3-98.2	94.3-98.6	-	-
B1	54.4-58.2	53.7-58.2	54.6-58.2	94.1-98	-
B2	55.1-60.7	55.4-59.5	55.0-60.7	80.2-85.8	99.2-91.4

Footnotes: Determined using pair-wise distance matrices created in the DNASTAR Megalign Program.

Table 4.10: Percentage amino acid identity within and between the different subtypes of the hMPV G protein.

Group	A1	A2	A1 Outliers	B1	B2
A1	85.6-100	-	-	-	-
A2	57.0-70.8	64.3-100	-	-	-
A1 Outliers	60.9-78.8	50.0-62.7	71.0-97.9	-	-
B1	30.7-34.8	29.0-35.8	29.4-35.8	90.5-98.7	-
B2	33.1-36.9	30.7-35.0 -	28.1-37.8	65.4-75.0	81.2-99.2

Footnotes: Determined using pair-wise distance matrices created in the DNASTAR Megalign Program.

4.4.3 Hydrophilicity profiles

To gain a better understanding of the effect of the amino acid differences on the potential structure, hydrophilicity plots of G proteins from the different subgroups were generated. All subgroups share a very similar hydrophilicity pattern with a region of hydrophobic residues at the N-terminus representing the cytoplasmic and transmembrane domains. The remaining two thirds of the protein is almost entirely hydrophilic, representing the exodomain. There is a small hydrophobic region in all subtypes between positions 104 to 110, highlighted by the arrow, which may represent an internal structure of the protein. The similarity in the hydrophilicity profiles indicates that the amino acids that differ between subtypes share similar physical and chemical properties properties.

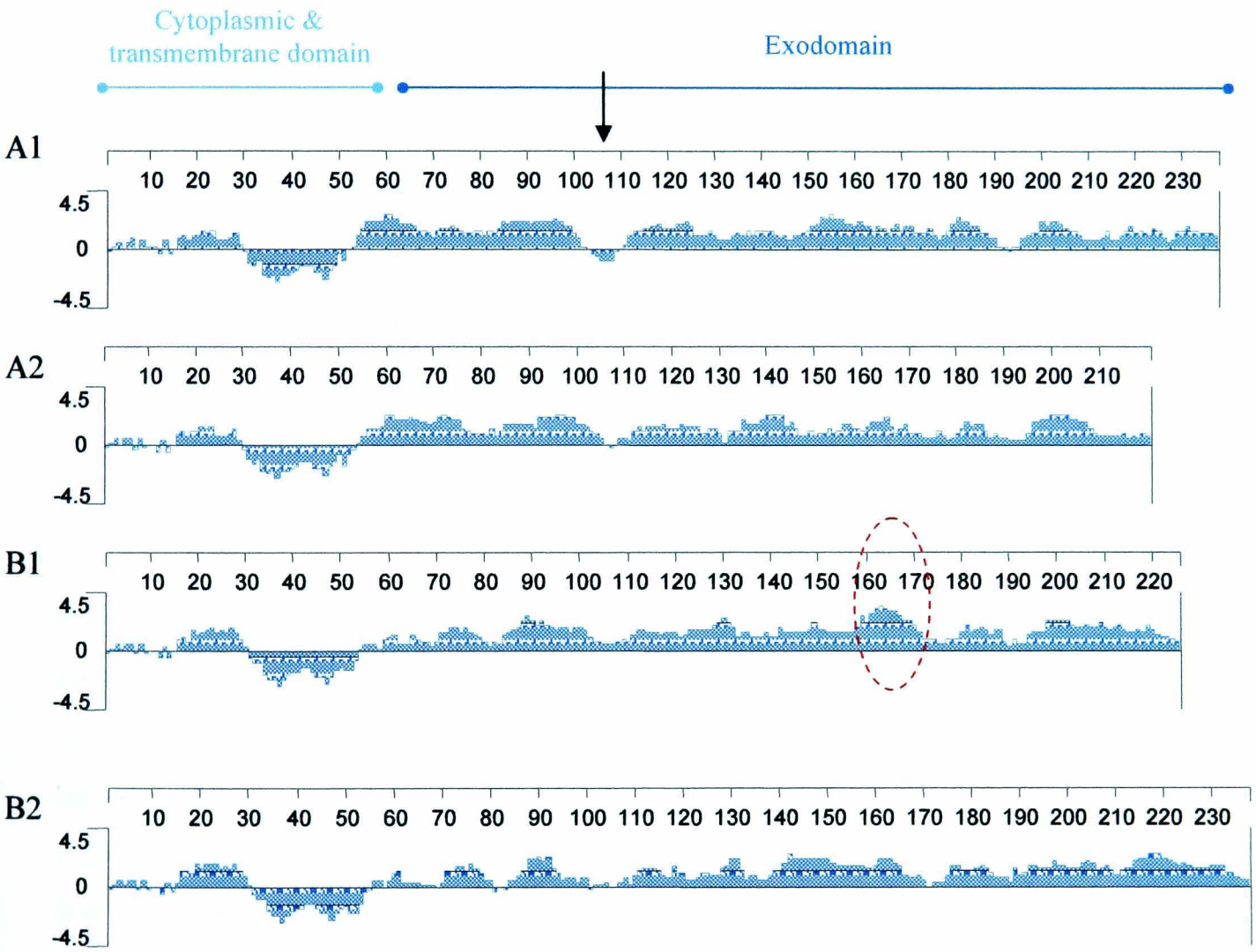


Figure 4.16: Hydrophilicity plots of hMPV G proteins from subgroups A1, A2, B1 and B2 produced using the DNASTAR Protean program using the Kyte-Doolittle method (1982). Hydropathy values were assigned for all amino acids and were averaged over window of 9 amino acids. Circled in red is the multi basic site insertion noted in the B1 strains.

4.4.4 Variation in protein length: Insertions and Deletions and Substitutions.

One of the notable features of the hMPV G protein is the variation in the length of the protein between and within different subtypes of the virus (Figure 4.15). Premature terminations codons may occur as a result of frame shift mutation due to insertion or deletion or due to nucleotide substitution. The length variations for the different subgroups are summarised in Table 4.11. The A1 subgroup contains proteins of 236 amino acids, the A1 outliers are treated separately so as avoid potentially skewing results, and amino acid lengths in this group range from 193 to 235 amino acids in length. The variation in the length of this protein has important structural and functional implications.

Table 4.11: Variation in the lengths of different G protein within and between subtypes.

Group	Length (aa)
A1	**236**
A2	**208 - 228**
A1 Outliers	**193 - 235**
B1	**202 - 232**
B2	**223 - 241**

With the exception of the sequences highlighted in yellow in the amino acid alignment above the A1 sequences have 2 different conserved protein lengths of 236 and 222 amino acids. Those with 222 amino acid lengths were all derived from one study, and only nucleotides 1-666 were deposited on gene bank. These sequences were not deposited with a publication and therefore it is unknown whether they represent the ORF of the strains that were sequenced or whether the sequencing method used stopped at position 666, therefore these should be disregarded when discussing gene length.

Within the A2 subgroup there are 3 conserved protein lengths of 216, 218, and 228 with the exception of sequence JMLEIC 1011 generated in this study discussed below. In the B2 subgroup, all but one sequence from the Netherlands; NL182 has a protein length of 236 amino acids. Strain NL182 is 17 amino acids shorter than the others and is a result of a C to T substitution at position 658. Within the B1 strains 3 different protein lengths are noted. All but two consist of 232amino acids. Sequence NL199 from the Netherlands contains 226 amino acids, and is the result of a G to T substitution giving a TAG

termination codon. Sequence JM RCGP348034 generated in this study contains 203 amino acids as a result of a frame shift mutation due to a G nucleotide insertion at position 570 leading to a premature TGA termination codon.

The amino acid alignment in Figure 4.15 highlights in red and purple possible insertions or deletions resulting in a frame shift mutation which stand out in particular as they result in proteins whose carboxyl terminal look very different to related strains that do not contain the mutation(s). In all cases these mutations have lead to premature termination of translation resulting in the production of truncated forms of the G protein lacking the carboxyl terminal as mentioned above.

The most notable is an insertion of 5 amino acid resides resulting in a multi basic region conserved in all the B1 subtype viruses. Analysis of the nucleotide alignment of this region shows an insertion of 15 nucleotides resulting in an adenosine rich region that is not present in any of the virus strains from other subtypes. The inserted nucleotides and subsequent amino acids are shown in Figure 4.17. This region relates to a highly hydrophilic region in the hydrophilicity profile of the B1 G proteins, as highlighted in Figure 4.16, and does not contain any glycosylation sites.

NT	C	A	A	A	A	A	A	G	G	G	A	A	A	A	A	G	G	A	A	A	A
AA	Q			K			R			E			K			G			K		

Figure 4.17: The inserted Adenosine rich nucleotide sequence at position 480-495 in the B1 subtype viruses and the corresponding Amino acids which result in the presence of a multi basic site within the B1 Strains and amino acid positions 160-165 highlighted in Figure 4.15.

The amino acid alignment in Figure 4.15 also boxes in purple a possible change in ORF in the A group sequences shaded in yellow, and A2 sequence JMLeic1011.

Analysis of the nucleotide alignment of this region shows an insertion of 2 adenosine residues in sequence JMLeic1011 at position 458 and 459 following a run of 8 consecutive adenosine residues. This insertion results in a frame shift mutation, which consequently leads to premature termination at a TAG stop codon at nucleotide position 624.

An insertion of a single adenosine residue at position 456 occurs in sequences JMLeic210, 13, and 2392, following a run of 6 adenosine residues. For sequences JMLeic210 and 2392 this insertion results in a premature TAG termination codon at nucleotide position 708. For sequence 13 a premature TGA termination codon occurs at position 624. The difference between the two stop codons is due to a nucleotide substitution at position 624 in the JMLeic13 sequence. It is not known without sequencing further clones or by population sequencing whether these are naturally occurring mutations, or whether these have arisen as a result of sequencing a single clone.

A cytosine deletion also occurred in sequence JMLeic1627 at position 449. This deletion also occurred amongst a run of 7 adenosine residues, resulting also in a change of ORF that leads further downstream to a premature TGA termination codon at position 624.

4.4.5 Glycosylation

The potential O (Figure 4.18) and N (Figure 4.19) linked glycosylation profiles of the different subtypes of G was also investigated due to the important role glycosylation is thought to play in the antigenicity of this and related proteins and its potential structure. The strains used for analysis are reference strains NL100GA1, NL1700GA2, NL194GB2 and NL199GB1.

The results show that as expected the hMPV G protein is predicted to be extensively glycosylated with O-linked sugars. Overall serine and threonone amino acids (receptor sites for O-linked glycosylation) account for approximately 32% of all the amino acids in the G protein. In accordance with the hydrophilicity profiles of the protein the majority of glycosylation occurs in the hydrophilic ectodomain. The glycosylation potential of the small number of serine/ threonone amino acids present in the cytoplasmic and transmembrane regions fall below the 0.5 threshold, and are therefore unlikely to be glycosylated. Most notably in B1 viruses there appears to be 2 distinct regions of glycosylation with the serine/ threonone amino acids around the 170 amino acid position showing a lower glycosylation potential than the remainder of the ectodomain. Less than 8% of the potential O-linked glycosylated sites are conserved between all 4 subtypes.

In contrast there is very little N-linked glycosylation with only 4- 5 predicted N- linked sites. The pattern of N-linked glycosylation varies considerably between subtypes with one conserved site occurring in the cytoplasmic- transmembrane region of the protein, and falls below the glycosylation potential threshold in A subtypes. Notably the glycosylation site found at position 52 is not present in the B1 and B2 strains analysed.

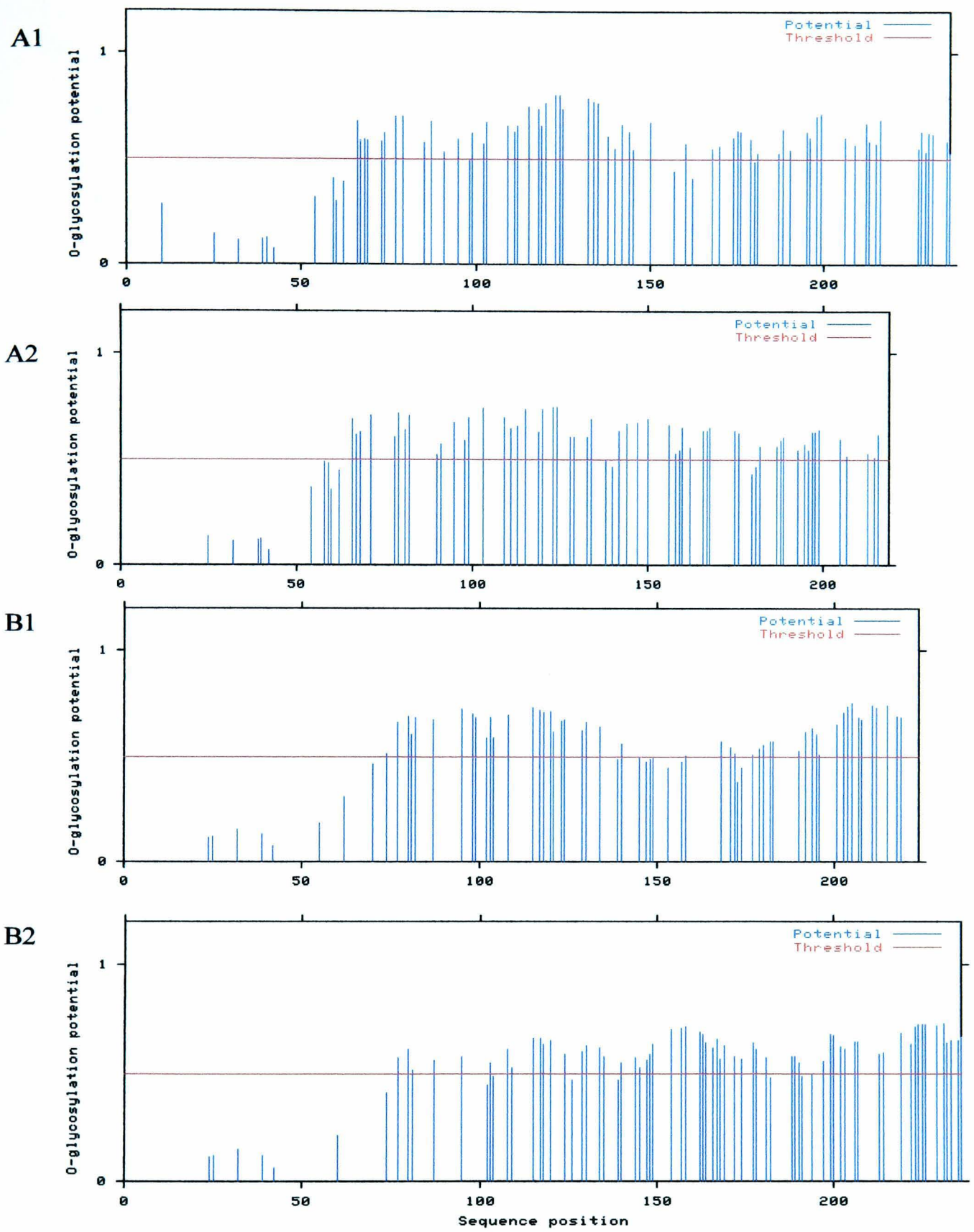


Figure 4.18: Predicted O-linked Glycosylation sites using the NetOGlyc 3.1 Server available at http://www.cbs.dtu.dk/services/NetOGlyc/. Blue represents the potential of glycosylation at all possible glycosylation sites. Red indicates the threshold above which glycosylation at that site is considered highly likely to occur. Prediction, conservation analysis and structural characterization of mammalian mucin-type O-glycosylation sites K. Julenius, A. Mølgaard, R. Gupta and S. Brunak. Glycobiology, 15:153-164, 2005.

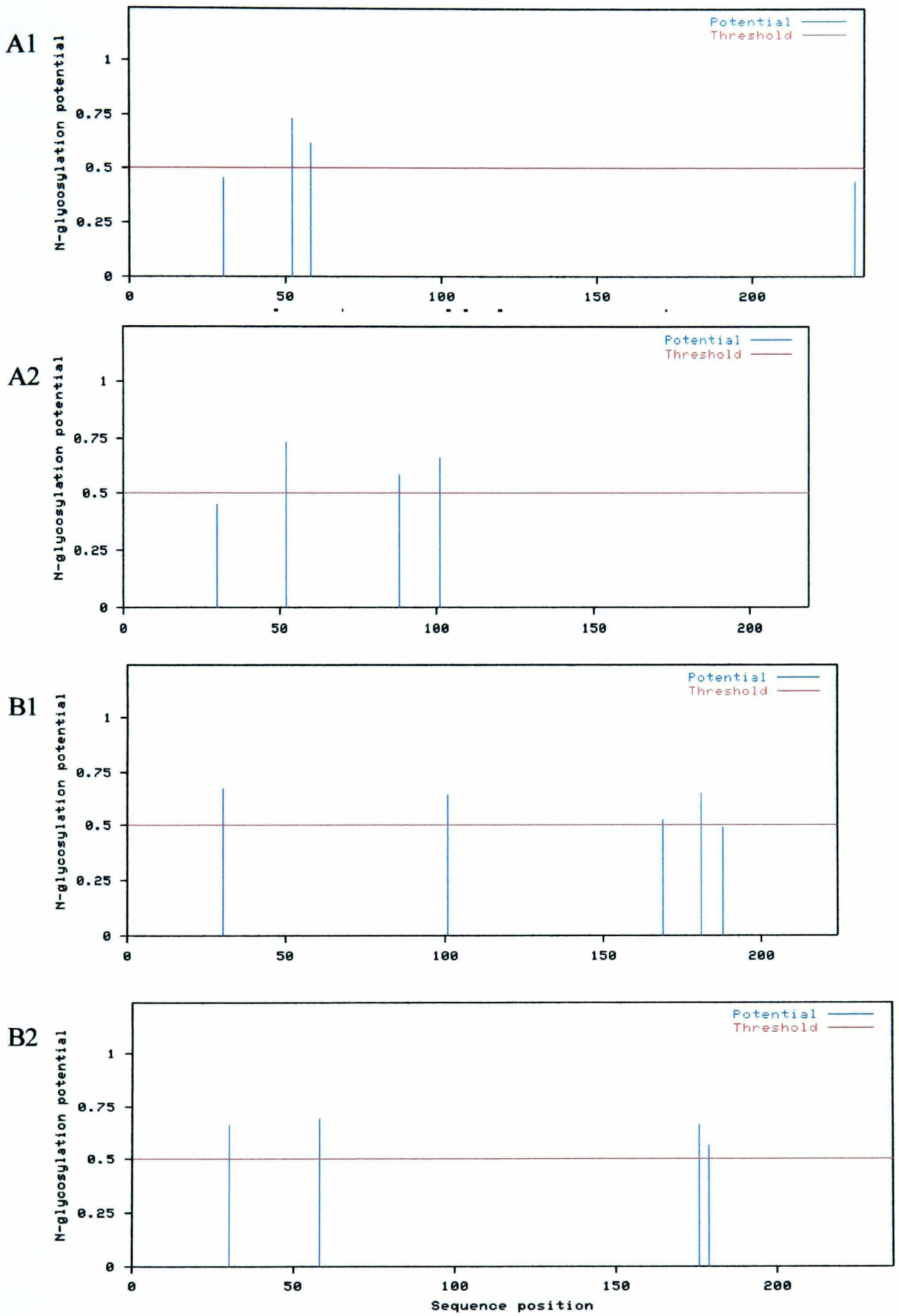


Figure 4.19: Predicted N-linked Glycosylation sites using the NetNGlyc 1.0 Server available at http://www.cbs.dtu.dk/services/NetNGlyc/. Blue represents the potential of glycosylation at all possible glycosylation sites. Red indicates the threshold above which glycosylation at that site is considered highly likely to occur. Prediction of N-glycosylation sites in human proteins. R.Gupta, E.Jung and S.Brunak. In preparation, 2004

4.5 Discussion

Reported here is the development of a sequencing strategy for the full-length open reading frame of the hMPV attachment glycoprotein, and its application to clinical material known to be positive for hMPV. Also reported is the phylogenetic and sequence analysis of the generated sequences to establish their relationship with each other and those deposited in the Genbank Sequence Database (See appendix 2).

4.5.1 hMPV G RT-PCR

The developed RT-PCR was designed to detect both subtypes of hMPV using sequences deposited on the Genbank Sequence Database. There are other published reports of primers which amplify the hMPV G gene or regions of it (Biacchesi et al., 2003, van den Hoogen et al., 2002). However most of those protocols require good quality samples for example isolates grown from clinical material, and as discussed previously it was not possible to culture hMPV from the clinical samples used in this study. Secondly many of the published primers were designed with fewer sequences, and many of the primers, especially those located within the gene would have been unsuitable due to the high level of nucleotide variation observed.

The RT step as discussed in chapter 3 was based on a random hexamer approach. The PCR itself was a hemi nested PCR incorporating a generic first round PCR followed by a subtype specific second round. The use of degenerate primers to capture both subtypes within the one PCR was investigated, but found to lack the sensitivity of the subtype specific second round PCR. A proof reading *Taq* polymerase was selected for this work to reduce any potential *Taq* polymerase errors, which may occur.

In this study only 30% of the F gene PCR positive samples analysed were also positive using the hMPV G gene PCR, indicating the G gene PCR is not as sensitive as the F gene. A number of reasons may contribute to this. Firstly, the material used for this PCR was predominantly the same cDNA material used for the initial F gene analysis, or nucleic acid re-extracted from the original material. Long term storage and multiple freeze thaw cycles increase the likelihood of sample degradation. The monitoring of such degradation could be achieved through the detection of housekeeping genes such as β-actin or GAPDH. The amplification of overlapping G gene fragments may also be another approach to generating hMPV G gene sequence where full length amplification of the gene may not be possible.

Secondly, the G gene primers designed using the limited number of hMPV G gene sequences available from the Genbank Sequence Database at the time, and as such it is likely that certain strain types were not amplified due to differences in one or more of the primer binding sites. The continually increasing availability of hMPV sequence will allow for the constant redesign and modification of primers to ensure the maximum numbers of strains are detected.

Thirdly, the G gene primer set was cross-reactive with human chromosomal material which reduces sensitivity through competitive binding of the primers. The redesign of primers or the use of a gene specific RT step may improve specificity. Alternatively, DNAse treatment of the total nucleic acid preparation to remove genomic DNA or the specific extraction of RNA could also be considered.

Fourthly, the differential expression of the F and G genes may result in different detection limits. As illustrated in chapter 1, the amount of mRNA transcribed for each gene depends on its proximity to the 3' end of the genome (Collins et al., 2001). As the F gene is located closer to the 3' end of the genome than the G gene, it can be assumed that more F gene mRNA would be present in the sample than G. The possibility exists that either; vRNA, mRNA or both were being detected. If mRNA was detected this may provide an explanation for the observed differences between the two assays.

Other differences that may influence the relative sensitivities of the two assays include the type of *taq* polymerase used, the processivity of the *taq* and the length of the amplicon, which for G was more than 3 times the length of that for F.

4.5.2 Sequencing of clones

The use of cloning in this sequencing strategy enabled hMPV G gene sequence from each sample to be generated, from which internal sequencing primers could be designed. The use of the TOPO TA cloning vector provided a fast and efficient cloning method, which did not require the use of restriction sites or blunt ended cloning which is often very inefficient. Sequence data generated from clones however has one inherent flaw, in that the species that is present in the clone may not be representative of the majority species in the viral population. Analysis of 10 different clones generated from 2 samples, indicated that quasispecies were present, but only one or two amino acid changes were observed in 2 of the 10 sequences indicating that a majority of the clones contained representatives of the

major species. The further analysis of clones should be carried out in order to address the level of variation within a viral population.

4.5.3 Analysis of strain diversity

Despite the fact that the detection rate of hMPV G was not as good as would have been liked, the 30 sequences obtained in this work account for 30% of all the internationally available full-length hMPV G gene sequences to date. Analysis of these sequences together with those available from the Genbank Sequence Database is reported here (Appendix 2).

As previously reported, phylogenetic analysis of G reveals that hMPV can be divided in to 2 lineages A and B, each with two sub-lineages (A1, A2 and B1, B2) (Bastien et al., 2003, van den Hoogen et al., 2004). Sequences from this study, which include samples from the community and hospital populations, were all A subtypes with the exception of 1 B strain detected in the community. Similar strains were found to circulate in both the community and hospital populations, which is not surprising, as the children investigated in the hospital study would most likely have acquired the infection whilst in the community. It was also noted that similar strains were detected in the different years of the study. This has also been noted in previous work by van den Hoogen et al (2004), who also reported that similar strains of hMPV were found to circulate in different countries (van den Hoogen et al., 2004). Similar strains of hRSV viruses have also been isolated at different time points, and in different geographical locations (Cane & Pringle, 1995).

Predominantly A strain viruses were recovered in this study which is reflected also in the distribution of all the available G gene sequences to date. It would be speculation to say whether this represents a predominance of A strains over B strains in this study given the number of samples which failed to generate sequence, or whether this represents differences in the sensitivity of different assays to the different subtypes.

Analysis of the predicted amino acid sequences of the G ORF revealed a high level of sequence diversity within and between subtypes (28-38 % amino acid identity between A1 and B1). This level of sequence homology is slightly different to that previously reported for hMPV G using different subsets of these sequences as would be expected with an increased number of sequences. Most recently; Peret *et al* (2004) has reported 74-100% nucleotide and 61-100% amino acid homology within the A lineage. 77-99% nucleotide and 63-99% amino acid homology Within the B lineage, and 52-58% nucleotide and 31-

35% amino acid homology between lineages (Peret et al., 2004). The higher rate of amino acid variation, compared with nucleotide suggests that the amino acid changes that are occurring are advantageous to the virus and supports the view that this protein evolved under immunological pressure.

This level of variation is greater than that observed in the hRSV for which 80-95% amino acid homology has been observed within subtypes, and 38% between subtypes(Garcia et al., 1994, Johnson et al., 1987). For Avian metapneumovirus a high degree of amino acid homology has been reported within subtypes (98.5-99.7 %), however between the A and B subtypes there is 38% amino acid homology (Juhasz & Easton, 1994). In another study looking at the variation in the G gene among avian metapneumoviruses an even greater level of sequence divergence was reported between the G proteins of the different metapneumovirus subtypes with only 5.6% amino acid identity between B and D, 7.2% between C and B, 10.1% between A and D, 12.6% between c and D, 13% between A and C , and finally 28.7% between A and D (Alvarez et al., 2003).

4.5.4 Selective pressure

Despite the amount of variation observed the short branch lengths of the trees presented in this thesis indicate there was very little progressive accumulation of amino acid changes, also observed elsewhere (van den Hoogen et al., 2004).While this relates to only the short time period for which hMPV isolates are available this contrasts with hRSV in which the accumulation of amino acid changes has been reported (Cane & Pringle, 1995, Woelk & Holmes, 2001, Zlateva et al., 2004).

The transition/ transversions (Ts/Tv) and non-synonymous/synonymous (Dn/Ds) ratios provide some information as to the selective pressure acting on G. It has been reported that the lower the value of the Ts/Tv, and the higher the value of the Dn/Ds ratios, the more evidence there is for positive selection (Hurst, 2002). This type of analysis has not yet been published for hMPV.

This study found very little difference in the Ts/Tv ratios between viruses circulating in the community and those in hospitalised children. This indicates that selective pressures are equal in both groups and supports the phylogenetic findings that similar strains where present in both populations. Of particular interest however was the marked difference in the Ts/Tv ratios between A and B subtypes. A greater number of transversions were

observed in the A dataset when compared with the B dataset. This suggests a greater selective pressure on A subtype viruses is occurring. Similarly transversions occurred more often in B1 viruses than B2.

As expected, there were a greater number of synonymous changes detected in all data sets investigated in this study. The greatest proportion of non-synonymous changes however occurred in A subtype viruses, indicating, as with the Ts/Tv ratios, that A subtype viruses may be under grater positive selective pressure, although the significance of this compared with B viruses was not established.

Overall the results indicate that despite the large degree of variation observed, within and between subtypes, the hMPV G gene is under negative selection, as during the short time period over which these sequences were acquired there has been no accumulation in amino acid changes. More temporal data is therefore required to assess whether amino acid changes are acquired over longer periods of time. Furthermore this data does not rule out the possibility that particular sites or positions within the gene are under positive selection as shown for RSV (Cane & Pringle, 1995, Woelk & Holmes, 2001, Zlateva et al., 2004).

Both the Ts/Tv and Dn/Ds ratios were determined from global alignments of the hMPV G sequences that included both conserved and variable regions of the gene. To identify whether different regions of the gene were under different selective pressures regional alignments comparing conserved and variable regions should be analysed. Furthermore to ascertain whether this type of selection is true throughout the entire hMPV genome TS/TV and Dn/Ds ratios should be calculated for different genes.

The differences observed in the selective pressure of A and B subtypes, may be occurring for a number of reasons, although given the limited number of sequences available it is not possible to ascertain what is actually happening. Firstly; the increased selective pressure on A subtype viruses may be skewed by the greater number of A sequences analysed compared with B. Secondly; the greater level of selective pressure on A subtype viruses may also be an indication of the predominance of A subtypes over B. With a larger proportion of the population perhaps having experienced A subtype infections the pressure on A virus to evade host immune responses may be greater than B. Alternatively the immune response elicited by A subtype viruses may differ from that of B subtype viruses in such a way that greater pressure is put on A viruses to adapt. Further research however

is required before conclusions can be made as to the significance and interpenetration of these results.

4.5.5 Mechanisms of change

The Pyrimidine Ts/ Purine Ts ratios indicate that the majority of transitions are occurring between the purine bases adenine (A) and guanine (G) in all data sets. This is consistent with the fact that approximately 40% of the gene is comprised of adenine bases and supports the view that this gene is constantly changing.

The bias in the high frequency of AG transitions has also been shown for a number of other RNA viruses; in particular RSV (Martinez et al., 1997), measles (Cattaneo et al., 1988, Wong et al., 1991), and influenza (Suarez-Lopez & Ortin, 1994). One mechanism which may explain the high rate of AG transitions observed in RNA viruses, involves cellular adenosine deaminases (Martinez & Melero, 2002). These enzymes are thought to act on small dsRNA structures, such as those formed during transcription or replication. This enzyme converts adenine into inosine bases, which behave as guanine bases during base pairing (Schaub & Keller, 2002). Adenosine deaminases have been associated with the modification of cellular RNAs as well as hyper-mutational events within retrovirus and hepatitis genomes (Bass, 1997, Jayan, 2004, Schaub & Keller, 2002)

The most notable mutations observed in the hMPV G gene sequence were additions or deletions of nucleotides which resulted in frame shift mutations. These mutations significantly altered the amino acid content and length of the resulting proteins. Variation in length is also frequently observed in the hRSV G protein (Rueda et al., 1991). All the frame shift mutations observed in the G gene occurred in regions where runs of multiple adenine bases were present. This is a typical occurrence in adenosine rich templates and is thought to arise as a result of polymerase error due to a form of slippage at these sites (Hausmann et al., 1996, Jacques et al., 1994).

It is not known however whether these mutations observed in the A1 outliers group are naturally occurring or are as a result of sequencing only one clone. In study by Cane *et al,* 1993 it was demonstrated that frame shifting in the RSV G protein as a result of polymerase slippage at runs adenosine bases occurred in 14% of clones derived from mRNA, 58% of clones from genomic RNA, and 50% of clones generated from in vitro

transcripts (Cane et al., 1993). Further analysis of these strains is required, either through sequence analysis of multiple clones, or by population sequencing.

The possible insertion of 5 amino acids in the hMPV G B2 subgroup provides an interesting point for discussion. Major changes in the RSV G protein due to insertions of up to 60 nucleotides have also been reported (Trento et al., 2003) and provides another example of the drastic genetic alterations that can be tolerated in this protein. The insertion of these 5 amino acids into the hMPV B2 subtype G protein introduces a multi-basic site that is not present in the G proteins of the other hMPV subtypes. Multi basic sites in other viral proteins are associated with a protease cleavage, often required for protein activation and function; such as membrane fusion; for example in the influenza haemagglutinin or RSV and hMPV Fusion proteins (Bolt et al., 2000, Nagai et al., 1991, Stieneke-Grober et al., 1992). The presence of such sites in hMPV or RSV G proteins however has not been previously reported. Whether the presence of such a site in the hMPV G protein plays a similar role would be an interesting point for investigation.

4.5.6 Glycosylation

The O-linked glycosylation potential calculated in this study was based not only on the presence of serine /threonine residues but also on the predicted secondary structure and accessibility of the sites. The observed potential for extensive O-linked glycosylation in G was expected, and is comparable to that of RSV (Garcia-Beato & Melero, 2000, Lambert, 1988, Wertz et al., 1989). The majority of the potentially O-linked glycosylated sites occur in the hydrophilic ectodomain; however, less than 8% are conserved between all 4 subtypes. Furthermore, huge differences in the glycosylation potential of strains belonging to the same subtype were also observed (data not shown). For RSV G, variation in glycosylation has also been observed in the same strain grown in different cell types, and within the same cell type (Garcia-Beato et al., 1996). Glycosylation therefore is likely to contribute further to the extensive heterogeneity of this protein.

The high level of glycosylation is thought also to mask dominant immune-epitopes that may be present. However the analysis of the reactivity of RSV monoclonal antibodies with un-glycosylated RSV G species indicates that antibody recognition is often dependent on the presence of oligosaccharide side chains (Garcia-Beato et al., 1996, Melero et al., 1997).

As a result of the high level of glycosylation at serine and threonine residues and a high proline content the G proteins of hMPV and RSV have been likened to mucins, a group of proteins produced by mucosal epithelial cells which are essential for the mucociliary clearance of foreign particles present in the respiratory tract (Jeffery & Li, 1997). Abnormalities in either in the quality or quantity of mucins may result in the development of pathological airways as seen in chronic bronchitis, asthma, COPD and cystic fibrosis (Fahy, 2001, Kim et al., 1997). The similarity of hMPV G proteins to that of its host's may confer an advantage to the virus in terms of evading host immune responses, and may also contribute to the development of mucociliary clearance dysfunction. Many other viral glycoproteins have also been reported to contain mucin like regions for example Ebola and Crimean-Congo hemorrhagic fever virus glycoproteins (Sanchez et al., 2002, Yang et al., 2000).

The role of glycosylation in host immune response evasion has been widely reported; however the role of glycosylation in influencing the structure of G should not be overlooked. It has been reported that the extensive glycosylation observed in mucins may provide support and rigidity to these proteins which would otherwise have fairly flexible structures and incomplete glycosylation has been associated with severe pathology due to alterations in mucin structure and function (Ciborowski & Finn, 2002, Kim et al., 1997, Rose, 1992)

4.5.7 Protein structure

Secondary structure prediction of hMPV G indicates that a majority of the protein is comprised of turn and loop regions, with very little evidence for the presence of alpha helices. hMPV G is similar to that of RSV G, in terms of its hydrophilicity, and glycosylation profiles, however in terms of the tertiary and quaternary structure these two protein may differ significantly.

The variation in the length of hMPV G suggests that the far carboxy terminal of the protein does not play any structural role. Variation in the length of G has also been reported previously (Bastien et al., 2004, Ishiguro et al., 2004, van den Hoogen et al., 2004). As has variation in the length of RSV G (Martinez et al., 1999, Melero et al., 1997, Rueda et al., 1991).

A 3D structure of RSV G has been proposed (Melero et al., 1997). However, the lack of sequence homology between hRSV and hMPV G makes 3D structure prediction of hMPV G impossible using bioinformatic techniques. A number of noticeable differences between the two proteins also indicate these proteins may differ structurally. Differences include the presence of 4 cystine residues and a conserved region in the ectodomain of the RSV G protein both of which are not present in hMPV G. Firstly, the conserved region in RSV G is hydrophobic, and thought to confer an internal structure of the protein. Secondly, the 4 conserved cystine residues contain side chains which form 2 disulphide bridges, stabilising the structure and creating a cystine noose.

4.5.8 Protein function

The fact that the hMPV G protein appears to tolerate such dramatic alterations in its amino acid content, length, and glycosylation profile calls in to question the role G plays in the hMPV replicative cycle. Recently Biacchesi, *et al* (2004) demonstrated using infectious clones that hMPV viruses lacking the attachment glycoprotein grow equally as well in vitro as wild type viruses. Growth however was somewhat restricted in vivo (Biacchesi et al., 2004). Similar findings have also been reported for RSV in vitro; however deletion of G in vivo significantly inhibits virus replication (Techaarpornkul et al., 2001, Teng et al., 2001), indicating potential differences between the functions of these two proteins

Amino acid variation and truncation of the protein at the carboxyl terminal suggests there is little structural or functional hindrance in mutational occurrences. It is unlikely therefore that this region of the G protein provides a structural or functional role, but may be associated with the immune evasion strategies of the virus. Indeed the highly strain specific nature of antiserum generated against the G proteins of different hMPV strains supports this (Bastien et al., 2004).

The function of G in receptor binding can be studied in a variety of ways through the use of chimeric viruses or retroviral pseudotypes carrying the hMPV G protein. Epitope mapping and the use of monoclonal antibodies or compounds to block receptor binding in different cell systems could be used to determine the regions of the protein involved in receptor binding. It may also be possible to use oligosaccharide micro array technology to identify possible oligosaccharide moieties with which protein may interact.

The central conserved domain of the RSV G protein is thought to represent a possible receptor site against which neutralising antibodies are directed. A CXC motif present in this domain has been shown to interact with the CXC3R1 chemokine receptor (Tripp et al., 2001). The lack of this feature in the hMPV G protein may contribute to the differences observed in the inflammatory responses generated against hMPV and RSV as mentioned in chapter 1. The lack of a conserved region in hMPV G may also account for the inability to measure a meaningful neutralising antibody response against the hMPV G protein.

Furthermore a secreted version of the G protein is produced by RSV. It is thought this protein mops up antibodies targeted towards the G protein. It has also been demonstrated this protein retains its ability for receptor binding. It is possible a secreted form of hMPV G protein is also produced.

4.5.9 Future Work

The work presented in this chapter highlights many points for further investigation. What is the seroprevalence of hMPV in the community? What is the age of primary infection, and what does this suggest about the presence of maternal antibodies? What evidence is there for hMPV to cause repeat infections in the presence of antibody? What evidence is there that antibody recognition of G is affected by the large degree of variation observed in this gene, and the presence of glycosylation.

Chapter 5

Recombinant baculovirus expression of the hMPV nucleocapsid protein

5.1 Introduction

Estimation of the burden of disease due to a particular pathogen requires reagents that enable the assessment of the serological response to the virus; such reagents include viral antigen and virus specific antiserum. This chapter describes the development of such reagents.

Due to the limitations of producing antigen and antiserum with a virus that grows poorly in the laboratory, antigen was generated through the expression of hMPV proteins in a recombinant baculovirus expression system, against which hMPV specific antiserum was raised.

There are a number of systems available for the expression of heterologous proteins each with their own inherent advantages and disadvantages. Prokaryotic expression systems are a fast and effective way of producing large amounts of protein; however, they lack a number of posttranslational modifications. Where conformationally correct and accurately modified proteins are required, as for this work, eukaryotic systems have an advantage. Such systems include the yeast 2 hybrid, SV40, recombinant vaccinia, recombinant baculovirus and plasmid expression systems.

Mammalian cell expression using a simple plasmid system that utilises transcriptional regulatory elements offers an advantage over other mammalian expression systems. It is simple, safe, and offers moderate levels of gene expression in a wide variety of mammalian cell lines without the potential interference and added complication of other viral proteins or helper viruses required in other techniques. Depending on the features of the chosen vector stable cell lines constitutively expressing the protein of interest can be established. The moderate levels of protein expression however, are often insufficient for producing the large quantities of protein required for antiserum production.

The recombinant baculovirus expression system is one of the most extensively used systems for heterologous protein expression and also offers the advantages of a eukaryotic system producing biologically active proteins, with correct folding, disulphide bond formation, oligomerisation and other posttranslational modifications similar to that of mammalian cells whilst producing a much higher level of protein expression compared to that seen in mammalian cells.

Baculoviruses are a diverse group of viruses found exclusively in insects and for decades have been manipulated and utilised in pest control and more recently exploited as a powerful tool for heterologous protein expression. The most commonly used virus for this purpose is *Autographia californica* nuclear polyhedrosis virus (AcMNV).

The baculovirus replication cycle can be divided in to 2 phases, early and late. During the early phase virus particles are released from the infected cell by budding through the cellular membrane. During the late phase, however, viruses are occluded in crystalline protein structures comprised of the polyhedron protein. The generation of occluded viruses in an essential part of the natural replication cycle allowing the virus to survive in harsh conditions when released from the dying host and ensuring subsequent hosts are exposed to a large number of virus particles thus guaranteeing infection. During the occlusion process fibrillar structures composed of the p10 protein also accumulate and are thought to function in promoting cell lysis. These functions, however, become obsolete in virus passed in continuous cell culture, eliminating the need for the polyhedron and p10 proteins. These proteins are both produced at very high levels at the very late stages of infection and therefore one or the other, or both, can be replaced with a heterologous gene of interest. The proteins will therefore be expressed under the control of the *polh* or p10 promoters with no detrimental effect to the virus.

However, the late expression of the proteins occurs when a majority of host cell protein synthesis has been shut off and therefore the efficiency of posttranslational modifications may decline later in infection resulting in the production of heterogeneous protein products that differ in the extent of their posttranslational modifications. Complex N-linked glycosylation may also differ slightly from that in mammalian cells as insect cells may substitute less complex oligosaccharides.

There are numerous examples in the literature of proteins from related viruses being expressed in the recombinant baculovirus system; including the expression of pneumovirus nucleocapsid, fusion, small hydrophobic and attachment glycoproteins, which were successfully used for immunoassay development and protein characterisation (Buraphacheep et al., 1997, Levely et al., 1991, Luo et al., 2005, Pastey & Samal, 1998, Sullender & Britt, 1996). The formation of RSV nucleocapsid structures similar to those of RSV infected cells have also been observed by electron microscopy when expressed in the

baculovirus system (Bhella et al., 2002, Meric et al., 1994) indicating the correct folding and processing of the expressed proteins.

The nucleocapsid (N) of hMPV shows a high level of amino acid sequence homology of approximately 95% between the A and B subtypes (Bastien et al., 2003). Serological cross reactivity between A and B subtypes has also recently been reported (Hamelin & Boivin, 2005). Phylogenetic analysis has shown that hMPV is more closely related to avian Pneumovirus C (APV C) than hRSV, with N amino acid similarities of approximately 89% and 43% respectively(van den Hoogen et al., 2002). This relationship has also been demonstrated serologically with cross reactivity occurring between hMPV and APV C N, but not with that of hRSV (Alvarez et al., 2004).

The N proteins of the pneumoviruses are immunogenic, and are one of the most abundantly produced proteins during infection. Antibodies to N appear early in infection and predominate throughout (Buraphacheep et al., 1997, Samal et al., 1993, Westenbrink et al., 1989). These combined characteristics therefore make N an ideal target for the development of immunoassays for the specific detection of hMPV antibodies. HMPV and N specific antiserum would also be extremely useful reagents for monitoring viral growth in cell culture, and as a diagnostic tool. N was therefore chosen for expression in the recombinant baculovirus system.

This chapter describes the expression of the hMPV N protein and the generation, and evaluation of hMPV specific polyclonal antiserum as potential diagnostic or research reagents

5.2 Aims

- Expression of the hMPV full length nucleocapsid protein (N-FL) and N fragments in the recombinant baculovirus system as an alternative to cell culture produced hMPV whole antigen.
- Generate high titre hMPV specific polyclonal antiserum.
- To evaluate the potential use of the antigen and antiserum as tools for the assessment of serological response.

5.3 Recombinant Baculovirus System

The baculovirus 'Bacmid' vector used in this work was kindly provided by Professor Ian Jones from the University of Reading. The bacmid vector contains the entire genome of the prototype baculovirus *Autographia californica* m*ultiple nucleopolyhedrois virus* (AcMNV) with a deletion in the essential region of the *polh* locus without which the virus cannot propagate. The bacmid was linearised by restriction digest and co-transfected into SF9 cells with one of the transfer vectors described below (Table 5.1). The transfer vectors used encode the essential sequence deleted in the bacmid vector and the gene of interest.

Based on the expression of other paramyxovirus nucleocapsid proteins in the baculovirus system it was expected that the majority of the N-FL and N fragments would be expressed in the cytoplasm of the SF9 cells.

5.3.1 Construction of Baculovirus Transfer Vectors

The commercially available plasmid pTriEx-2 Neo (Novagen) was used as the baculovirus transfer vector, and mammalian expression vector for N-FL and N fragments

The pTriEx-2 Neo vector is a multiple expression system capable of expressing in *E.coli*, Insect and mammalian cells. For expression in insect cells the vector contains the baculovirus p10 promoter, and transcription start sequence. Baculovirus sequence flanks the promoter and cloning site to permit the generation of recombinant baculoviruses. Expression in mammalian cells is mediated by a hybrid promoter composed of the CMV immediate early enhancer fused to the chicken β-actin promoter. The vector also offers an N-terminal 6-histidine tag, utilised here for the detection and purification of the expressed hMPV proteins.

The isolate UK324-02 was chosen as starting material, as it was one of the few clinical samples to grow in cell culture at a detectable level by RT-PCR. It originated from a 4-year-old male diagnosed with bronchiolitis and upper respiratory tract infection in 2002, collected as part of the investigation into the cause of illness in children hospitalised with ARTI (Chapter 3). This isolate belongs to the A1 subgroup of hMPV (Chapter 4).

RT-PCR on total nucleic acid extracted from the original clinical sample was used to amplify the full-length hMPV N (N-FL) and N fragments using primers containing the

appropriate restriction sites shown in Table 5.1 and 5.2 and shown schematically in Figure 5.2. The amplified PCR product was first cloned in to the pCR2.1-TOPO TA cloning vector as described previously, and positive clones analysed by restriction digest and sequencing of purified plasmid. The TOPO constructs and the pTriEx-2 Neo plasmids were digested with the appropriate restriction enzymes, the correct fragments gel purified, and ligated together using T4 DNA ligase. The ligated plasmid was transformed in to chemically competent *E.coli* and individual colonies screened by restriction digest, and sequencing of purified plasmid. Figure 5.3 shows the map of the resulting plasmids.

To aid in the detection and purification of the expressed proteins each gene was cloned in frame with a 6-histidine tag, supplied in the vector. The tag was placed at the N-terminal of the proteins followed by a short linker and then the protein or protein fragment. The 6-Histidine tag was placed at the N terminal to aid the ease of cloning, and also because it had the least effect on the structure of the protein when analysed using secondary structure prediction software. Figure 5.1 shows the predicted secondary structure, hydrophilicity plot and surface probability of the hMPV N-FL with and without the N terminal 6-histidine tag using DNASTAR protein prediction software. The results show that there is no difference in the predicted secondary structure, hydrophilicity or surface prediction plots between the two in the N region indicating that the tag and linker are not likely to have an effect on the folding, processing or solubility of the resulting protein. Furthermore the 6-histidine tag is strongly hydrophilic and therefore likely to appear on the surface of the protein allowing easy detection and purification.

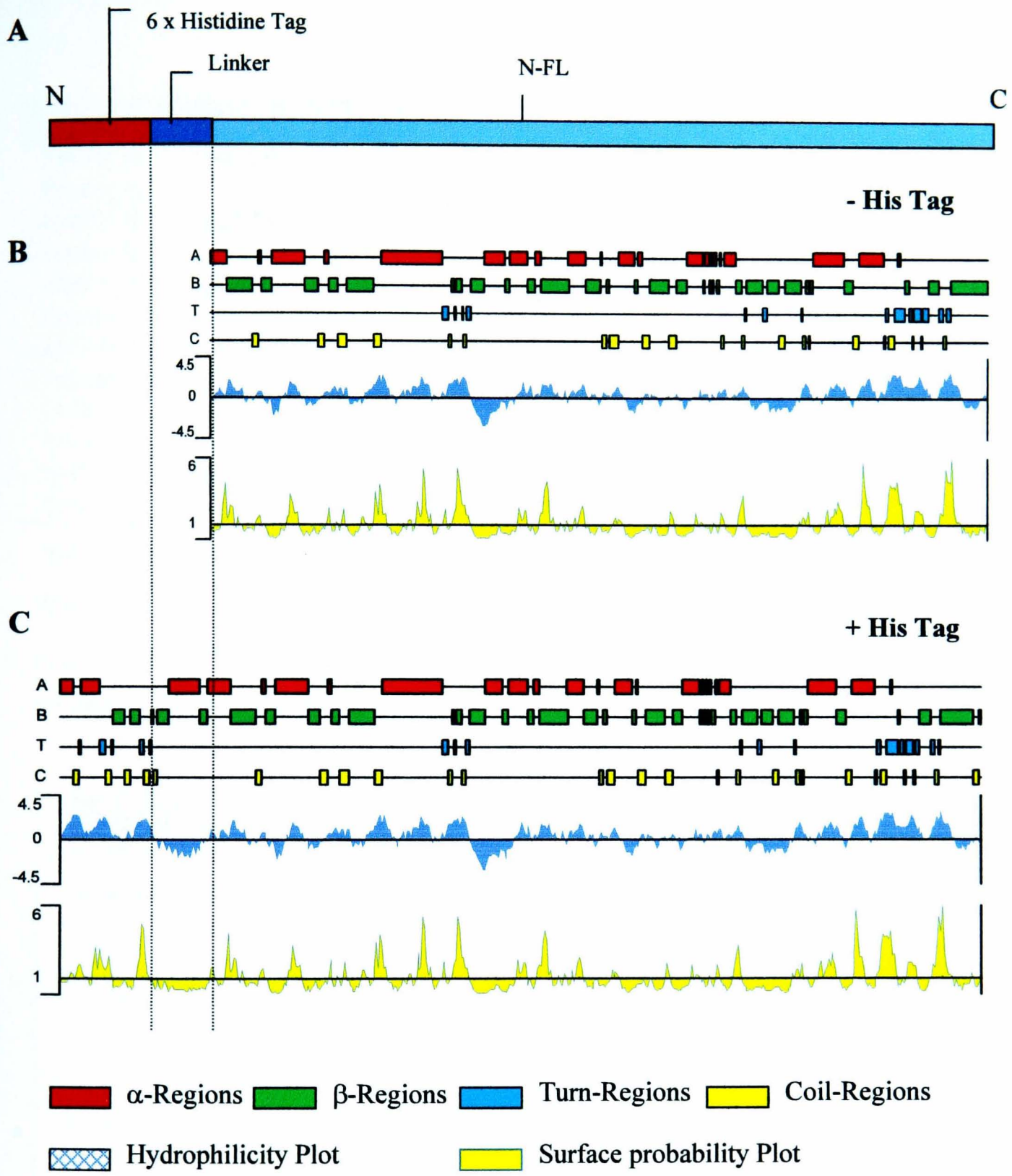


Figure 5.1 Secondary structure protein prediction of the hMPV N-FL with and without the 6-Histidine Tag and linker using DNASTAR protean. (A) Schematic showing the localisation of the Tag and linker, (B) secondary structure prediction of the N-FL without the 6-histidine tag, (C) Secondary structure prediction of the N-FL with the 6-histidine tag. α, β, Turn and coil regions were predicted using the Garnier-Robson method (Garnier et al., 1978). Hydrophilicity plot was predicted using the Hydropathy – Kyte-Doolittle method (Kyte & Doolittle, 1982), and the Surface probability predicted using the Emini method (Emini et al., 1985).

Primer	Position*	Sense	Sequence 5'-3'
hMPV N Primer A	40-59	+	GGATCCCATGTCTGTTCAAGGGATTCACC
hMPV N Primer B	520-502	-	GCGGCCGCCTTAGATTATGGGTGTGTCTGG
hMPV N Primer C	520-538	+	GGATCCCTTATTATGTGTAGGTGCC
hMPV N Primer D	880-859	-	GCGGCCGCCTTAACCTTGGACGGATACATG
hMPV N Primer E	880-899	+	GGATCCCGAGTTAAAACAGGTCACAG
hMPV N Primer F	1225-1206	-	GCGGCCGCCTTACTCATAATCATTTTGAC

Table 5.1: Primers used for the amplification of N-FL gene and N fragments for cloning into the pTriEX 2 Neo vector.

Footnotes: (*) primer positions according to reference strain NL00-1. Restriction sites for cloning are underlined. For schematic representation of primer location see Figure 5.2.

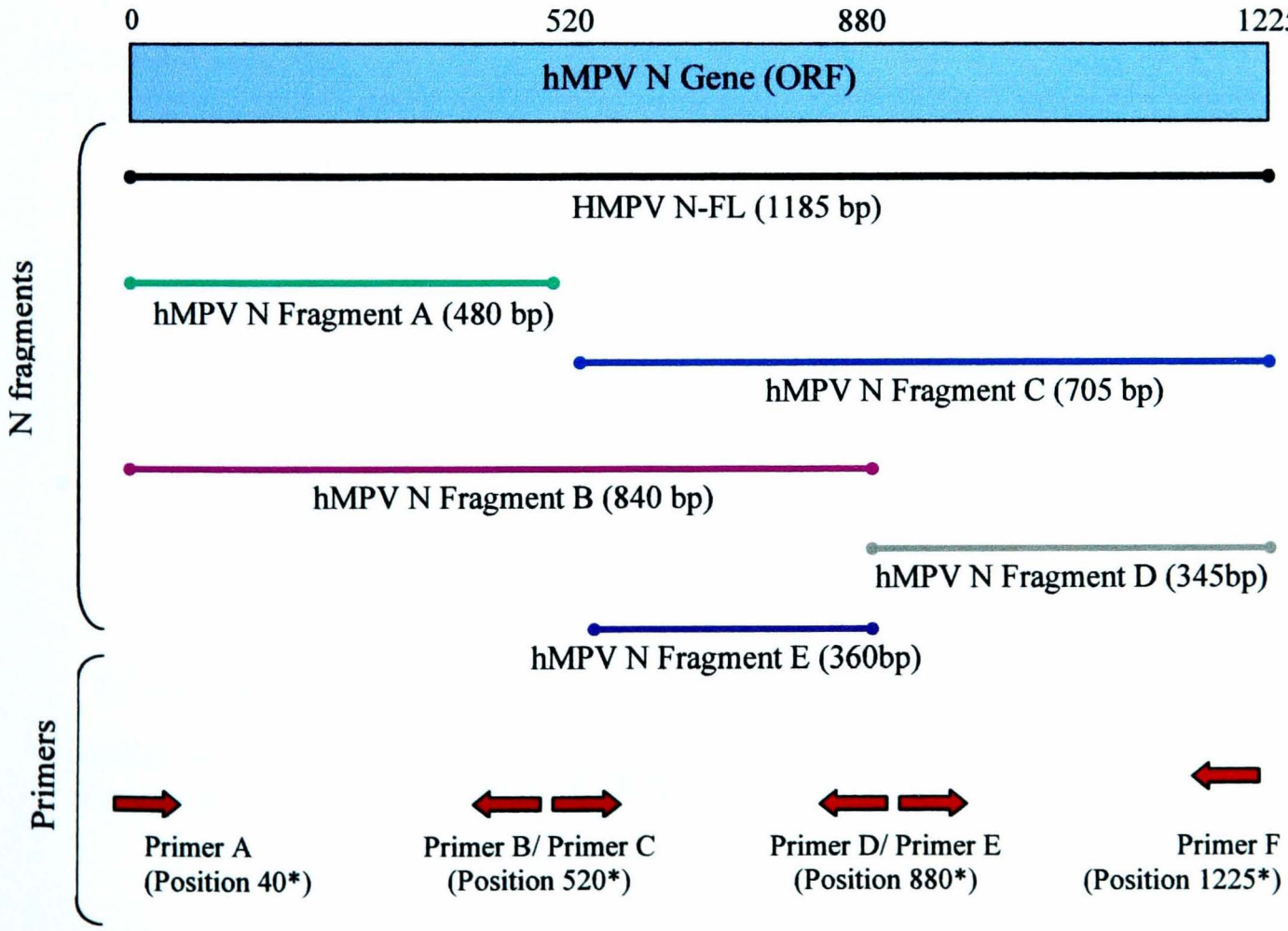


Figure 5.2: Schematic representation of N-FL and N gene fragments expressed in the recombinant baculovirus system and primer positions. The blue box represents the hMPV N gene ORF. (Black) N-FL, (Green) N Fragment A. (Maroon) N fragment B, (Blue) N fragment C,

(Grey) N fragment D, and (Purple) N fragment E. The size of each N region amplified in indicated in base pairs (bp) below the coloured line. Primers are represented by red arrows with the name of the primer below and the nucleotide starting position. (*) starting primer positions according to reference strain NL00-1. For primer sequences see Table 5.1.

Table 5.2 : Summary of the properties of the amplified hMPV N gene and gene fragments, transfer vector and expected protein size.

	Summary of properties					
Gene / Fragment	**N -FL**	**A**	**B**	**C**	**D**	**E**
Forward Primer	hMPV N A	hMPV N A	hMPV N A	hMPV N C	hMPV N E	hMPV N C
Reverse Primer	hMPV N F	hMPV N B	hMPV N D	hMPV N F	hMPV N F	hMPV N D
Expected Size (bp)	1185	480	840	705	345	360
Restriction Sites	BamH1/ Not 1					
Expression Vector	pTriEx-2 Neo					
Estimated Recombinant Protein Size (KDa) *	51	24.4	37.5	32.6	19.4	19.9

Footnotes: (*) Protein size estimated from amino acid sequence using EditSeq, DNAStar Lasergene software.

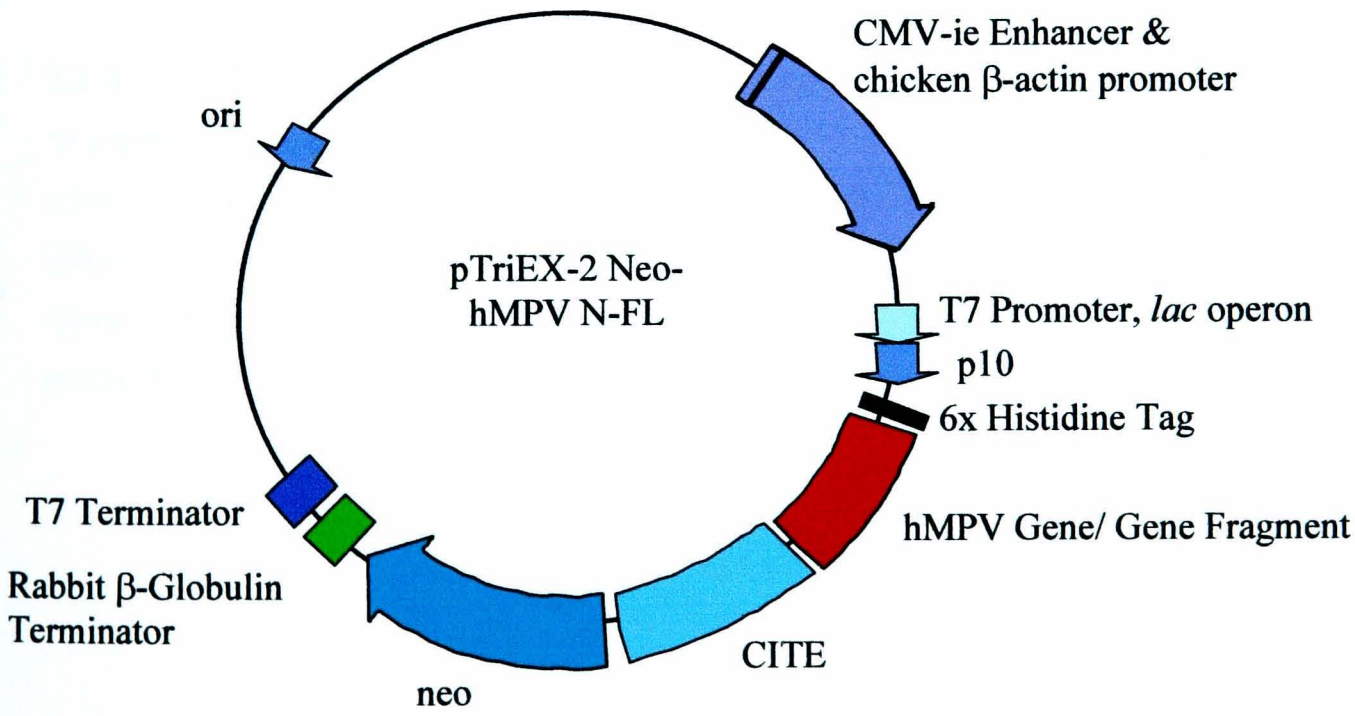


Figure 5.3: Schematic representation of the hMPV N-FL pTriEx transfer vector. Each construct contains a CMV ie enhancer and chicken β-actin promoter for expression in mammalian systems (purple), a T7 promoter for bacterial expression (pale green), and the p10 promoter for

recombinant baculovirus expression (pale blue). A 6x histidine tag (Black) is available just upstream of the multiple cloning site. The position of the inserted hMPV gene is shown in red. The vector contains the Rabbit β-globulin (green) and T7 terminator (dark blue). The constructs also contain the neomycin resistance gene for the selection of stable mammalian cell lines which operates under the control of the EMCV-derived Cap-Independent Translation Enhancer (CITE).

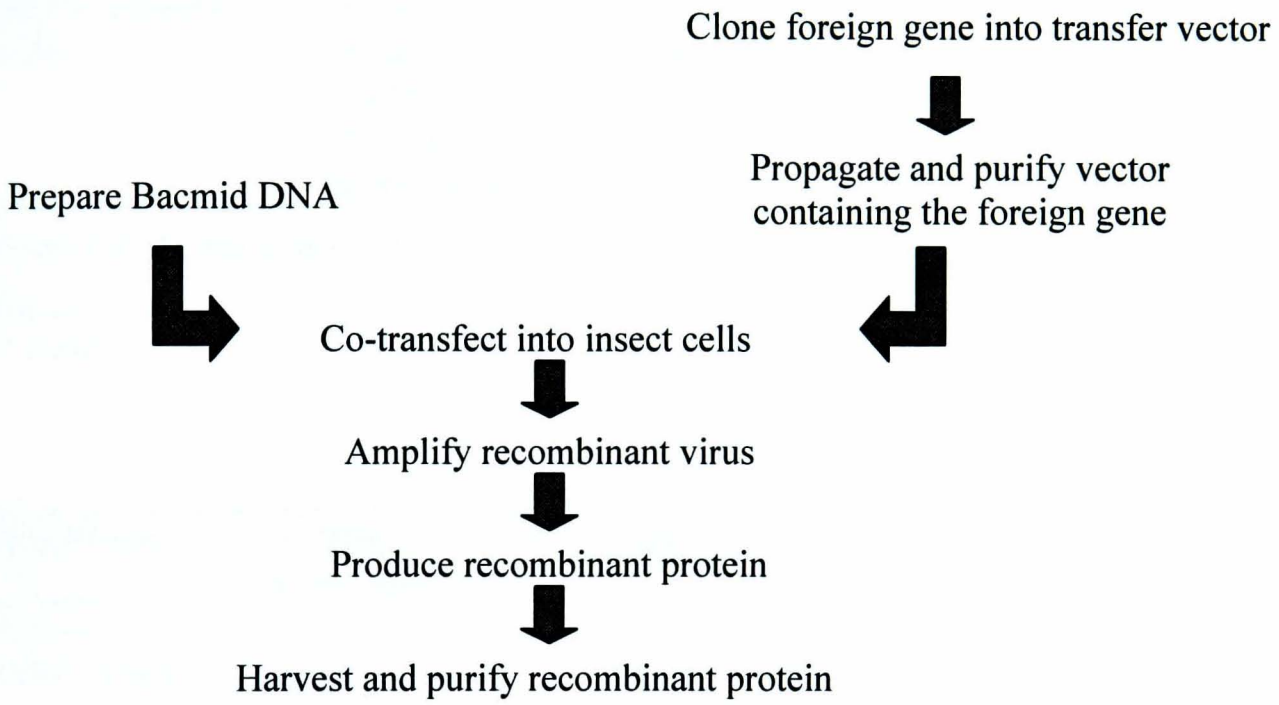


Figure 5.4 Flow diagram showing the major stages involved in producing protein in the recombinant baculovirus expression system. Propagate the transfer vector containing the foreign gene using competent cells and purify plasmid DNA. Co-transfect the bacmid DNA and recombinant transfer vector into *Sf9* insect cells. Amplify the resultant recombinant virus in *Sf9* insect cells. Use the amplified viral stock to produce protein. Purify your protein using Ni-NTA purification system

Antigen	Source	Lineage
N-FL	4-year-old male diagnosed with bronchiolitis and upper respiratory tract infection in 2002, collected as part of the investigation in to the cause of illness in children hospitalised with ARTI in the UK isolated in LLC-MK2 cells.	A1 (UK324-02) *
hMPV infected cells	Northern Ireland HPA (2004) investigation in to the cause of respiratory illness first isolated in RMK cells. Further propagated at Colindale HPA in LLC-MK2 cells by Dr Alison Bermingham.	A1 (NI1-04) and B1 (NI2-04)

Table 5.3: Details of the hMPV virus strains used as antigen in this chapter.

Footnotes: (*) see Chapter 4 for positioning in phylogenetic tree and alignment described as hMPV G clone.

Antiserum	Source of antiserum	Source of Antigen	Lineage
Rabbit α-hMPV Total	Colindale HPA raised against an hMPV infected RMK cells lysate isolated by Dr Joanne Stockton.	From child < 5 years collected as part of the investigation in to the cause of illness in children hospitalised with ARTI in the UK.	A (UK241-01)
Rabbit α-hMPV N-FL	This study, raised against recombinant baculovirus expressed hMPV G(s)-Fc High 5 cell culture supernatant	Recombinant baculovirus expressed hMPV N-FL generated in this study. See table 5.3 for further detail.	A1 (UK324-02) *

Table 5.4: Details of the hMPV specific rabbit serum used in this chapter.

Footnotes: (*) see Chapter 4 for positioning in phylogenetic tree and alignment described as hMPV G clone.

5.4 hMPV N Recombinant Baculovirus Expression

In order to make recombinant baculoviruses expressing the N-FL or N fragments the different transfer vectors were co-transfected with the linearised bacmid into SF9 cells and incubated for 6 days at 28^0C. The supernatant from the transfected cells was removed and used to inoculate fresh SF9 cells to grow up viral stocks. The cell lysates were analysed by Western Blot to confirm expression of the recombinant proteins (Figure 5.5).

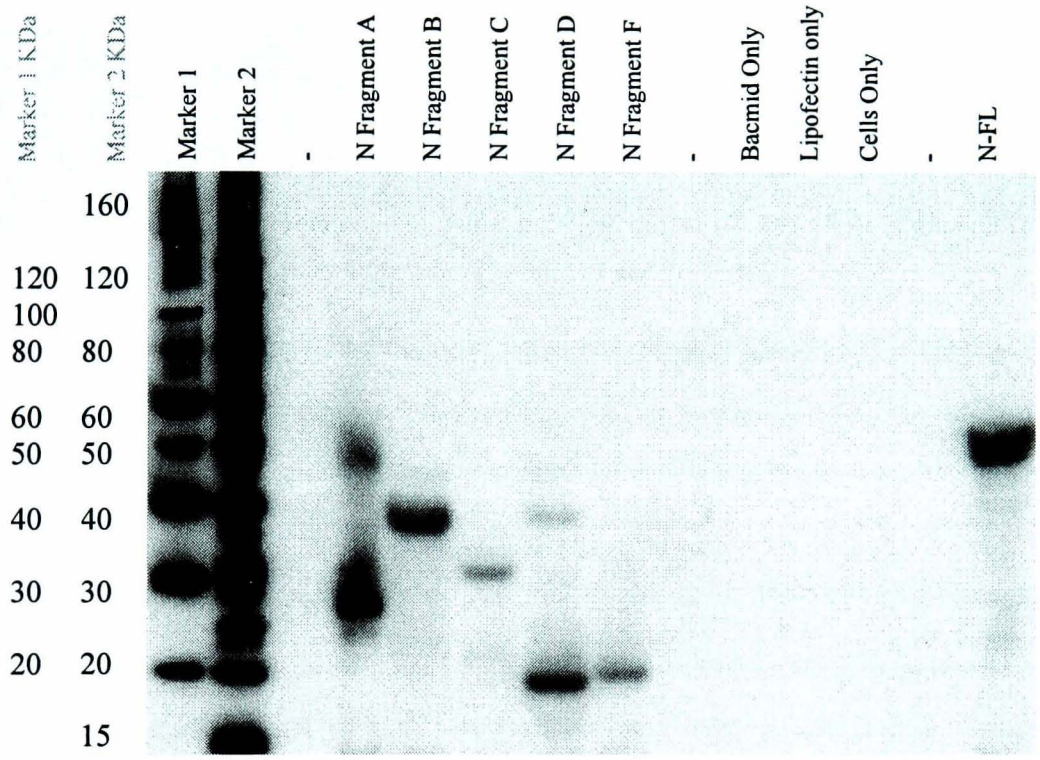


Figure 5.5: Western Blot analysis of co-transfected SF9 cell lysate of hMPV N Fragments A-E, N-FL, Bacmid only, lipofectin only and cells only controls harvested 6 days post transfection. Recombinant proteins were detected using a mouse α-6-histidine MAb (1:1000) and detected with a α-mouse IgG HRP conjugate (1:10000) by ECL reagent and autoradiography.

Western Blot analysis clearly shows that the 6 recombinant baculoviruses expressing the hMPV N fragments A-E and N-FL generate proteins of the expected size. In the lanes containing Fragments A and D higher molecular weight bands equating approximately to the correct size for protein dimmers are present. The different levels of expression are due to the fact the co-transfected cell lysate material was used for this blot and represents differences in the quality or amount of recombinant virus generated.

The baculovirus stocks were titrated by plaque assay to determine the number of PFU/ml of virus, an example of which is shown in Figure 5.6. Typically recombinant baculovirus hMPV N-FL reached high titres of around $2x10^8$pfu/ml. However, the recombinant baculoviruses expressing the hMPV fragments A to E grew only to titres of $1x10^6$pfu/ml, more than 2 logs lower than that of N-FL.

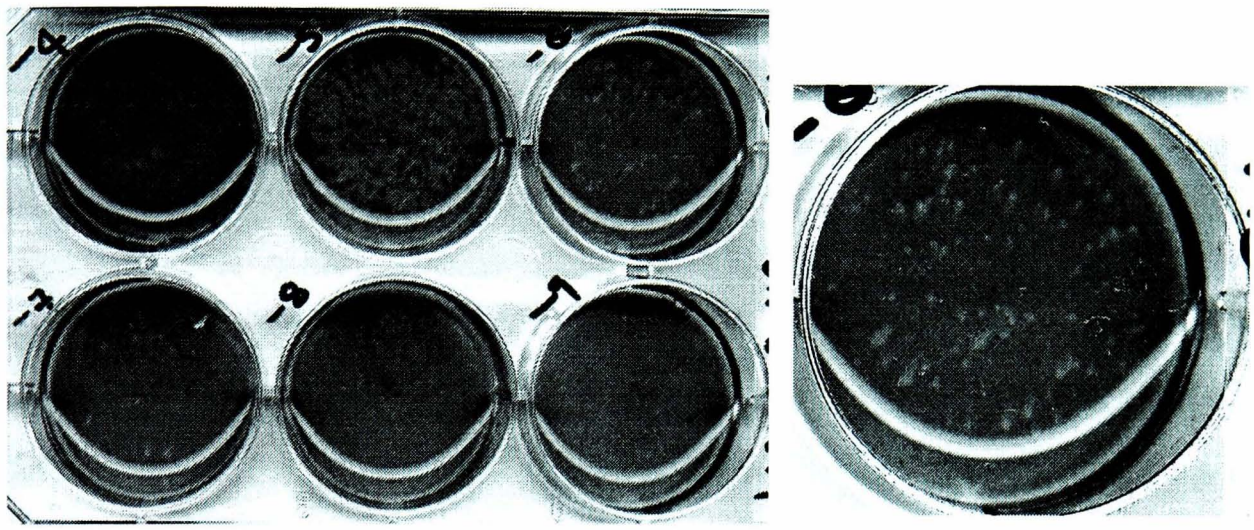


Figure 5.6: Example of a recombinant baculovirus plaque assay. Sf9 cells were infected with a ten fold dilution series of recombinant virus from 10^{-4} to 10^{-9} and overlaid with 1.6% agarose. At 7 days post infection cells were stained with neutral red and the plaques counted by eye.

To determine the optimal MOI for expression and day and for harvesting the expressed proteins, suspension cultures of SF9 cells were infected at an MOI of 3, 5 or 10 and 1ml removed from the culture flask every 24 hours over 5 days. The cells were pelleted and the lysate analysed by Western blot analysis to determine which time point yielded the largest amount of N/ml of cells. The Western blot results for the time course assay of N-FL at the optimum MOI of 3 is shown in Figure 5.7.

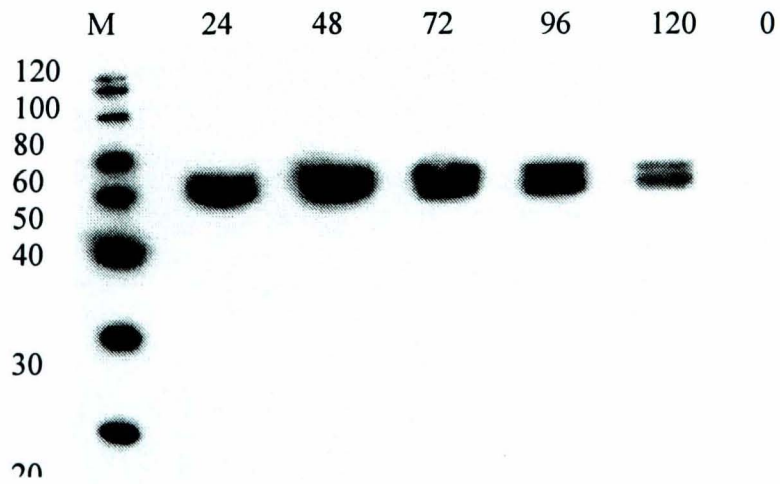


Figure 5.7: Time course assay of hMPv N-FL expression in the recombinant baculovirus system. Expression of N-FL in SF9 cells taken every 24 hours post infection. The expressed protein was

detected using a mouse α-6-histidine MAb (1:1000) followed by α-mouse IgG HRP conjugate (1:10000) with ECL reagent and autoradiography.

The results show that at 24 hours post infection N-FL is already expressed at reasonable levels. 48 to 72 hours post infection gives the optimal level of protein expression, which decreases from this point onwards, as host cell machinery shuts down and cells undergo apoptosis. At 5 days post infection a doublet is visible on the blot, which may represent different N-FL species with different levels of posttranslational modification. 72 hours post infection was therefore the optimal time for harvesting N-FL.

Recombinant baculoviruses expressing hMPV N fragments A-E however did not grow as well with virus titres more than 2 logs lower than that achieved for recombinant Baculovirus hMPV N-FL. The level of expressed protein was also considerably lower; despite attempts at optimisation of the culture conditions through varying cell seeding density and MOI (Data not shown). An MOI of 15 or greater was required to generate detectable levels of expressed protein by Western blot and therefore without virus purification the volume of virus required was not feasible. Virus purification was not possible however, due to time constraints.

5.5 Purification of Recombinant hMPV N-FL

The recombinant N-FL was purified under non-denaturing conditions to maintain the correct structure and conformation of the protein using a Ni-NTA Affinity chromatography system. The protein was purified in order to generate an hMPV N specific polyclonal antiserum.

Sf9 cells were infected with recombinant baculovirus hMPV N-FL and incubated for 3 days. Cells were collected by centrifugation and lysed. The lysed cell preparation was clarified by centrifugation and applied to the Ni-NTA bind slurry and incubated on a shaker at 4^0C for 2 hours. The slurry contained Ni-NTA sepharose beads and 10mM imidazole buffer. The 6-histidine tag of the recombinant hMPV N binds to Ni^{2+} cations immobilised on sepharose beads by nitro acetic acid (NTA). The imidazole present in the buffer competes with other Ni^{2+} binding proteins and histidine residues to reduce the amount of non-specific binding to the beads. The mixture was then packed in to a column for the washing and elution steps. Unbound and non-specific proteins were removed using increasing concentrations of imidazole buffer. N-FL was eluted using a 250mM imidazole buffer. Each of the fractions was collected and analysed by ELISA, Western blot and protein gel (Figure 5.8 A, B and C) for purity of the purified protein fragments, and assessment of the purification procedure.

The results show that there was still a large amount of N-FL present in the flow through that was not bound to the Ni-NTA beads suggesting the quantity of antigen used exceeded the binding capacity for the column size and quantity of Ni-NTA beads used. Only a small amount of hMPV N was removed from the column during the washing process and all non-specific, unbound protein was removed. A majority of N-FL was eluted in the first 2 elution fractions, with very little N-FL present in the 4th fraction. The protein gel however shows a number of contaminating bands in each of the elution fractions, which are most evident in elution fraction 2. These extra bands are also visible by Western blot analysis using a mouse α-6-histidine MAb followed by rabbit α-mouse IgG HRP conjugate. It is possible therefore that the highest molecular weight contaminating band is an hMPV N-FL dimer and lower molecular weight bands degradation products, despite the use of reducing conditions in the gel. It should not be overlooked however that other contaminating proteins with a high affinity for Ni^{2+} binding might also be present in the protein

preparation, the binding of which was not inhibited with the current concentration of imidazole.

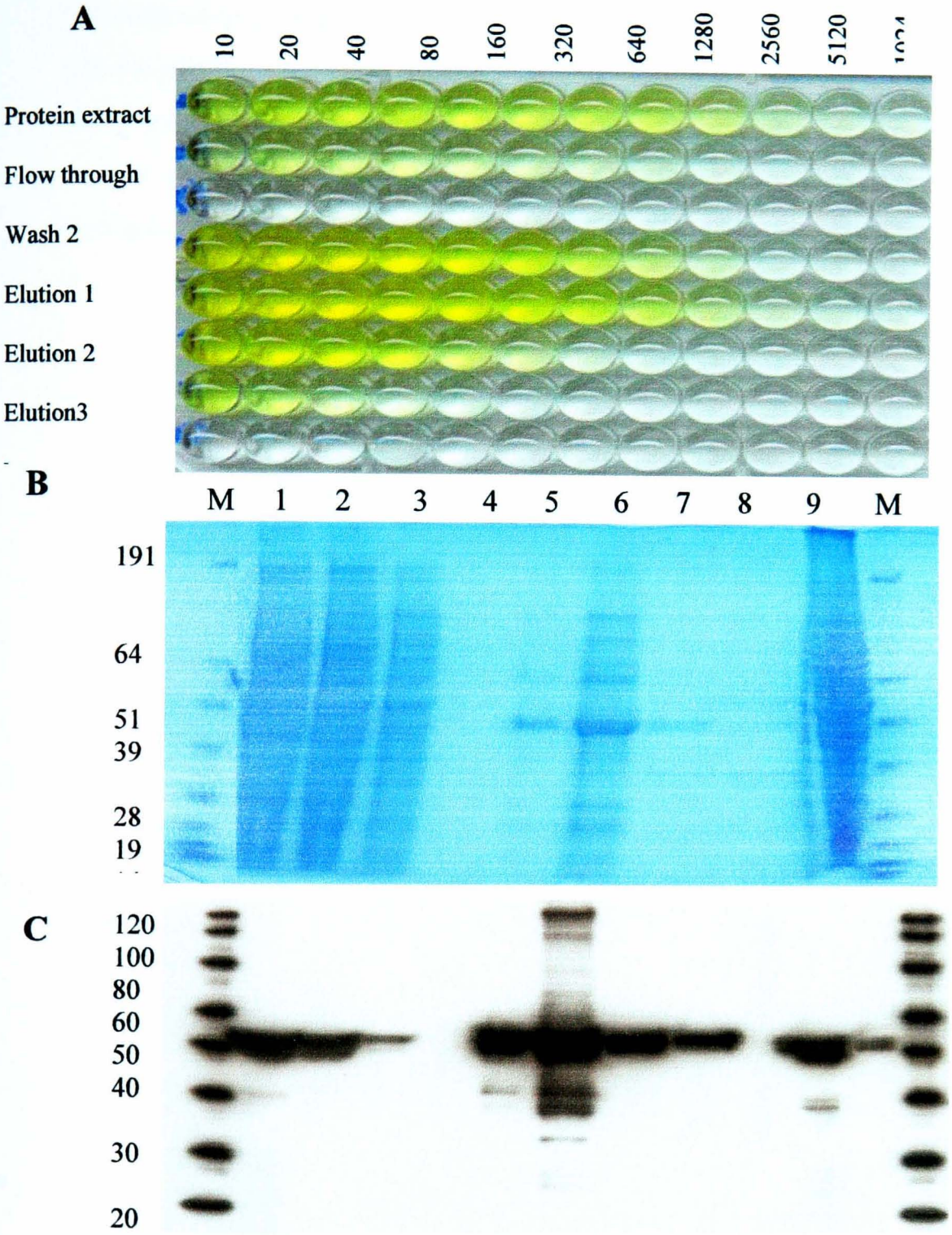


Figure 5.8: **(A)** Analysis of hMPV N-FL Ni-NTA protein purification. 96 well plates were coated with 100µl of the different fractions in 2 fold dilutions across the plate starting at 1:10 with 10mM PBS and incubated at 37^{0}C for 15 minutes. Plates were blocked with 1%BSA for 1 hour at 37^{0}C. N-FL was detected using the mouse α-6-histidine MAb at 1:1000 followed by Rabbit α-mouse IgG HRP conjugate with TMB substrate. The absorbance was measured at 420nm. Analysis of N-FL Ni-NTA protein purification by. (B) Protein gel analysis with Coomassie stain (C) Western Blot analysis using mouse α–6-histidine Mab 1:1000 followed by rabbit α- mouse IgG HRP conjugate 1:2500, and detected using ECL reagent and autoradiography. 10µl of each of the purification fractions was analysed. (M) Marker, (1) Protein Extract, (2) Column flow through, (3) wash 1, (4) wash 2, (5) Elution 1, (6) Elution 2, (7) Elution 3, (8) Elution 4, (9) positive control – N-FL SF9 cell lysate

By increasing the concentration of imidazole in the binding buffer to 20mM the amount of contaminating proteins were reduced (Figure 5.9). The amount of purified N-FL required for the rabbit innoculations required several batch purifications, and it is worth noting that there was a conciderable difference between the quantity and the purity of the purified protein preparations between batches. Thefore each batch was assessed by commassie staining of protein gels, and only the purest elution fractions pooled.

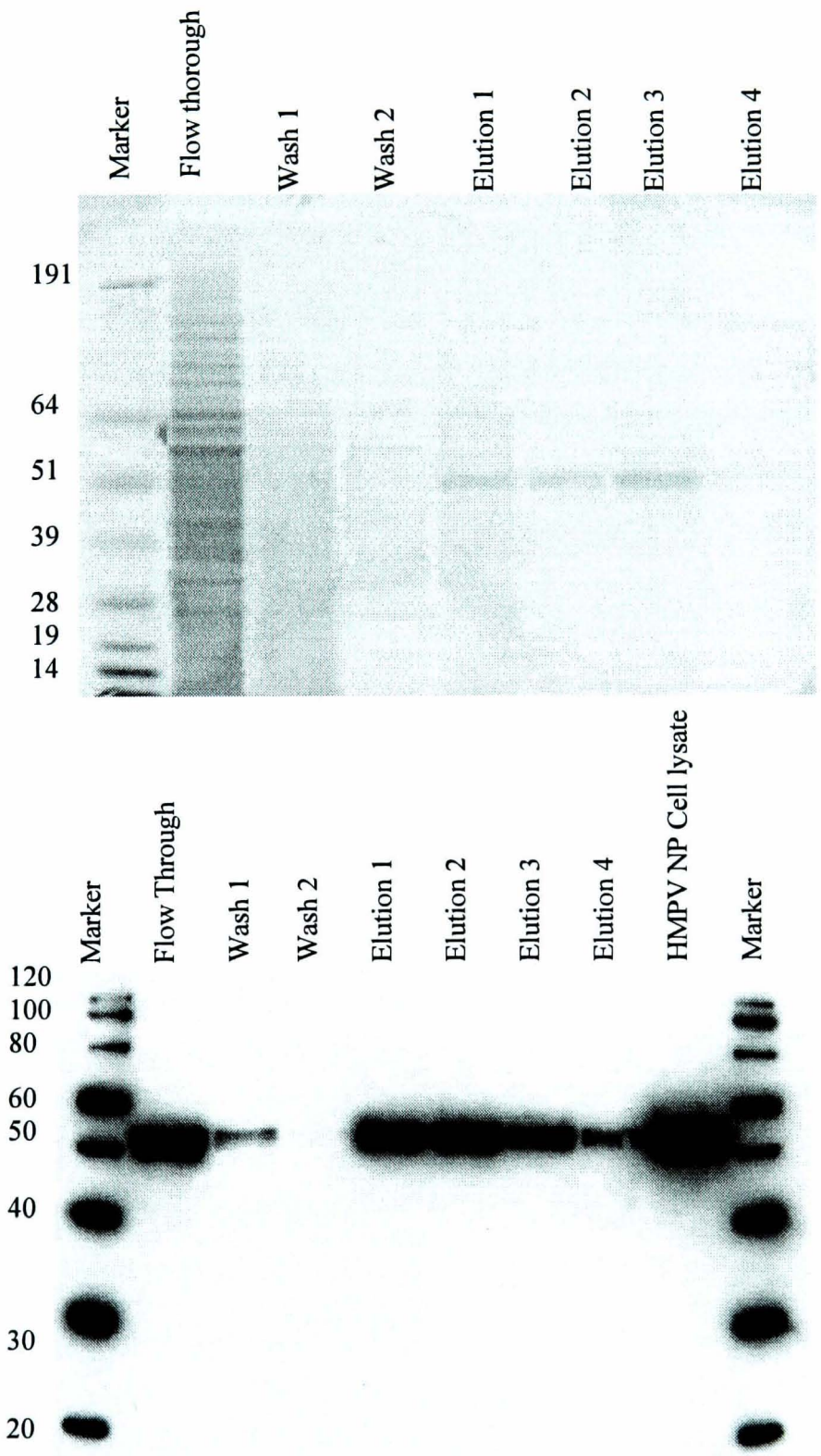


Figure 5.9: Analysis of N-FL Ni-NTA protein purification with increased imidazole concentrations. (A) Protein gel analysis with Coomassie stain (B) Western blot analysis using mouse α–6-histidineMab 1:1000 followed by rabbit α-mouse IgG HRP conjugate 1:2500, and detected using ECL reagent and autoradiography.

Analysis of the pooled purified protein prep by spectrophotometery and total protein assay gave an estimate of 0.74mg/ml of total purified protein. By Coomassie blue staining the proteins separated on a SDS PAGE gel along side a BSA standard marker (Figure 5.10) it was calculated using density analysis software that N-FL accounted for approximately 6.5% of the total protein preparation and was the major species in the preparation, this equates to a concentration of 47.4μg/ml of the purified preparation.

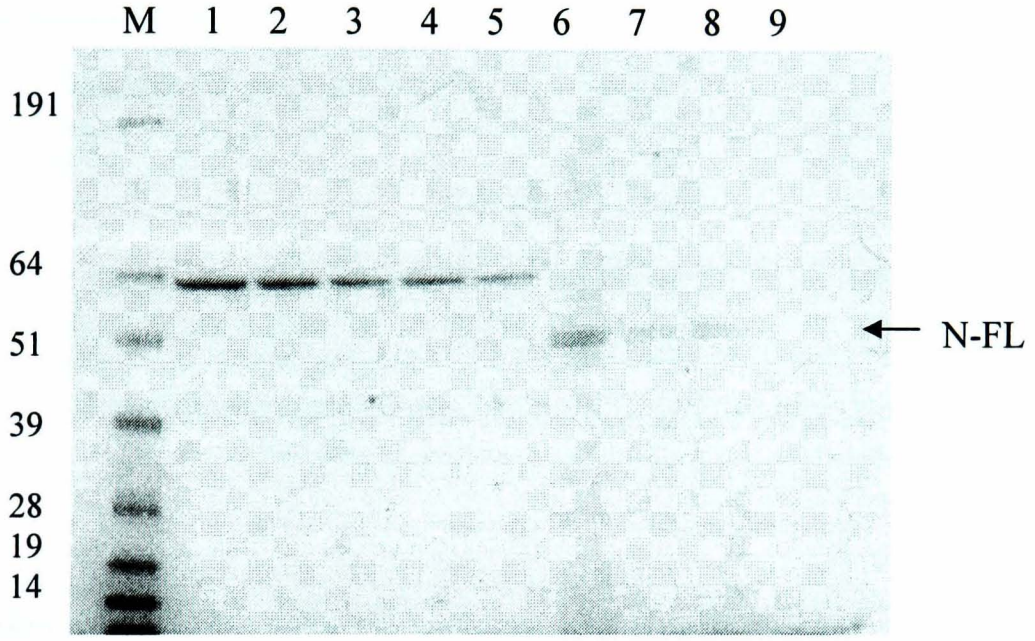


Figure 5.10: Protein gel analysis of hMPV N-FL purified protein preparation. M is a mobility marker; lane 1 to 5 is a BSA standard of 120ng, 90ng, 60ng, 60ng and 30ng respectively. Lanes 6 to 9 are different volumes of the purified N-FL preparation of 10ul, 5ul, 5ul and 2ul respectively. Gels were stained with Coomassie blue stain and analysed using a BioRad density imager and software.

5.6 Generation and Evaluation of hMPV Specific Antiserum.

For this work hMPV specific antiserum was raised against hMPV virus or recombinant baculovirus expressed hMPV N-FL and evaluated for their use as potential research or diagnostic reagents (Table 5.5).

Table 5.5: hMPV Specific antiserum raised and evaluated in this study.

Antiserum	Immunogen
Rabbit α-hMPV Total	hMPV infected RMK cell lysate approximately $1x10^5$ cells/ml. Unknown amount of hMPV virus, assumed to be very low titre, no adjuvant was used. 4 x 0.25ml of the inoculum was injected at 4 points along the dorsal of the rabbit with a booster given on day 30 into the hind leg muscle. Terminal Bleed taken day 60.
Rabbit α-hMPV N-FL	Purified baculovirus expressed hMPV N-FL 100ng of purified protein 10mM Purified LPS 0.5ml incomplete Freund's adjuvant. 4 x 0.25ml of the inoculum was injected at 4 points along the dorsal of the rabbit.100ng purified N-FL with 10mM LPS and saline was given as a second immunogen on days 21 and 40 into the hind leg muscle. Test bleeds taken days 35 and 55. Terminal Bleed taken day 60.

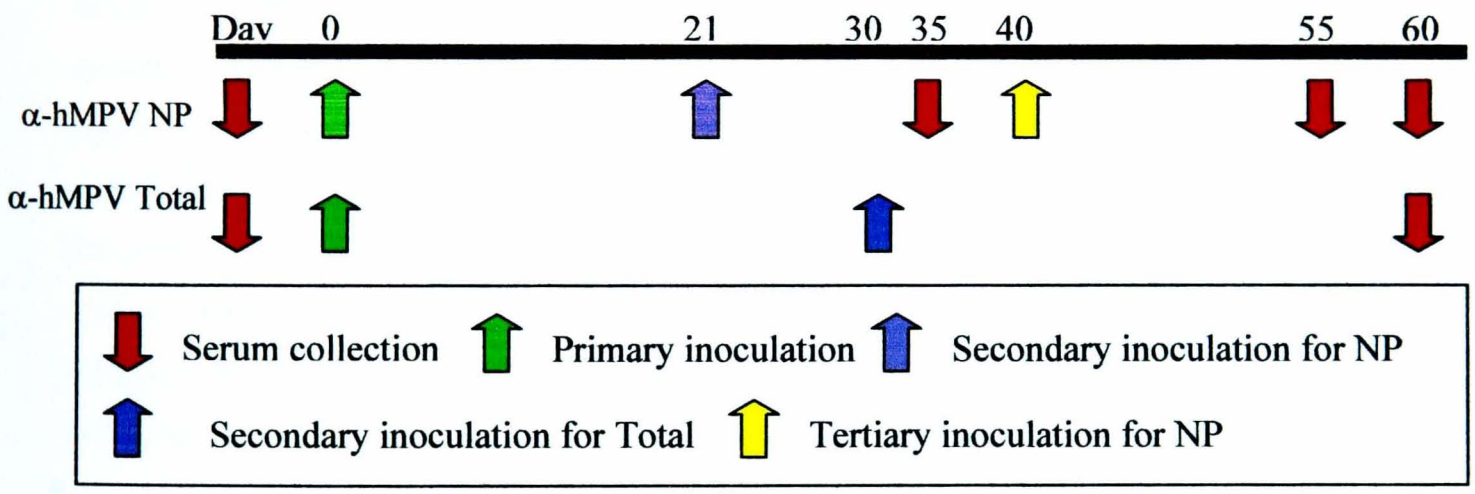


Figure 5.11: Schedule of rabbit inoculations for the generation of hMPV specifc antiserum. hMPV N-FL Specific polyclonal serum was raised against 100ng of hMPV N-FL mixed with purified lipopolysaccharide (LPS- prepared from *Escherichia coli* 0157 [kindly supplied by Dr Henrik Chart at the HPA Colindale]) and incomplete Freund's adjuvant to form an emulsion. Rabbit α-hMPV Total was generated by Dr Joanne Stockton at the HPA Colindale. Red: rabbit bleeds, Green: inoculation.

The sera were analysed by ELISA, Western blot, and immunofluorescence to evaluate their use as potential diagnostic or research reagents.

5.6.1 Analysis of hMPV Specific Sera by ELISA against recombinant hMPV proteins

The terminal bleeds for rabbit α-hMPV N and α-hMPV Total were tested by ELISA in a dilution series against the baculovirus expressed N-FL in the SF9 cell lysate with uninfected cells as a measure of background reactivity (Figure 5.12).

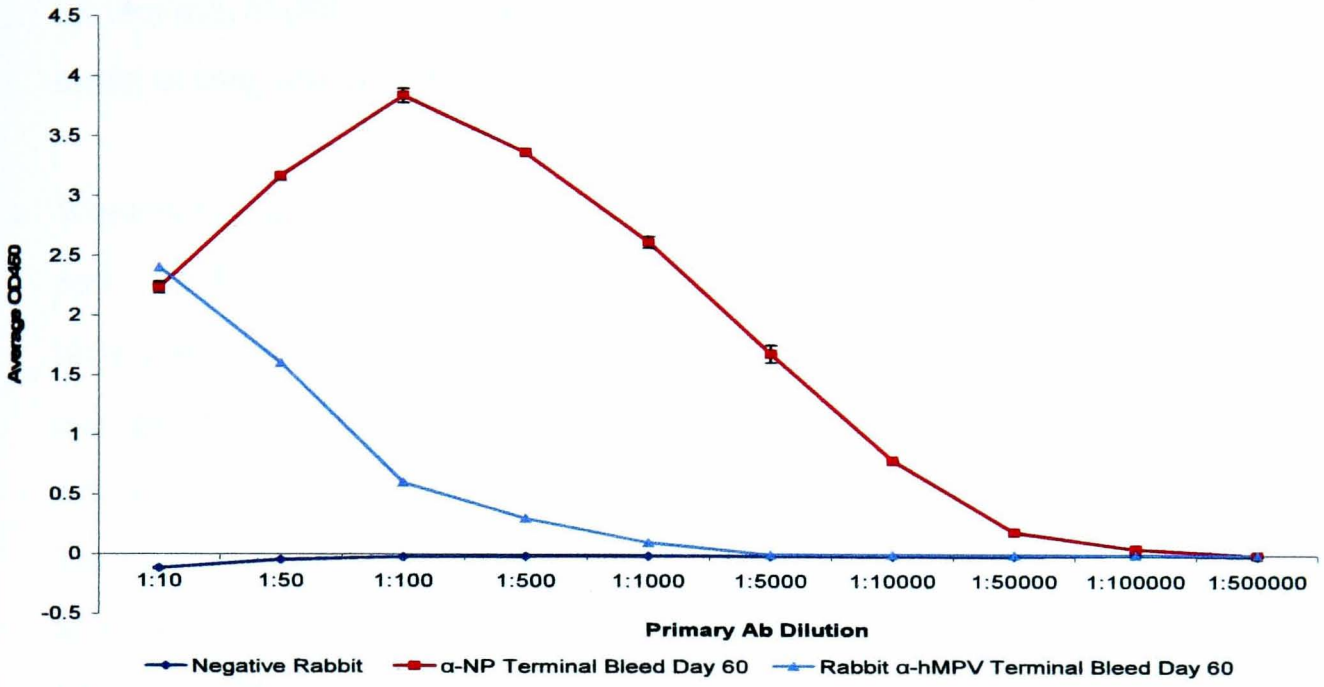


Figure 5.12: Analysis of hMPV specific rabbit sera by indirect ELISA against recombinant baculovirus hMPV N-FL infected or uninfected SF9 cell lysate. Error bars show +/- 1 Standard deviation. 96 well plates were coated with approximately 0.8µg of total protein of Baculovirus hMPV N–FL infected /uninfected cell lysate standardised by cell equivalence of $1.5x10^4$cells per well diluted 1:100 with 10mM PBS and incubated at 37^0C for 30 minutes. Plates were blocked with 5% milk for 1 hour at 37^0C. The hMPV specific Rabbit sera were tested in a dilution series from 1:10 to 1:500,000 followed by Goat α-Rabbit IgG HRP conjugate 1:2500 and detected using TMB substrate. The absorbance was measured at 420nm reference 620nm, and the negative cell lysate OD value was subtracted from that of the positive to give the corrected OD values.

Results show that both the Rabbit α-hMPV N and rabbit α-hMPV total sera recognised the N-FL antigen, although it is clear that the rabbit α-hMPV total has a considerably lower N antibody titre than the rabbit α-hMPV N-FL with only 1:10 to 1:100 dilutions reacting. The α-hMPV N-FL terminal bleed (Day 60) showed a high hMPV N antibody titre with dilutions of 1:50,000 still being reactive. The pre-bleed rabbit serum (negative) was negative at all dilutions tested.

Sera were tested by Western blot and immunofluorescence analysis at dilutions appropriate for the antibody titres determined by ELISA (Rabbit α-hMPV N-FL at 1:100, rabbit α-hMPV Total 1:50).

5.6.2 Analysis of hMPV Specific antiserum by Western blot using recombinant baculovirus expressed hMPV proteins

Sera were tested against cell lysate or supernatant positive or negative for recombinant baculovirus hMPV N-FL (Figure 5.13). The molecular weight species recognised by the serum in comparison with mouse α-6 –histidine MAb is summarised in Table 5.6.

Western blot analysis of hMPV N positive and negative SF9 Cell lysate taken 72 hours post infection shows that both the rabbit α-hMPV Total and rabbit α-hMPV N-FL recognise baculovirus expressed hMPV N-FL. While rabbit α-hMPV Total gives very little non-specific binding, the rabbit α-hMPV N-FL, at the dilution tested, also recognises a number of other species. These species however are not detected by the mouse α-6-histidine MAb indicating that the extra bands detected by rabbit α-hMPV N-FL are likely to be due to cross-reactivity with cellular proteins as oppose to degradation products of N-FL. The hMPV N-FL (51KDa species) is however the predominantly recognised species and reactivity against the cellular proteins was reduced by cross absorption with uninfected SF9 cells, and further dilution of the serum.

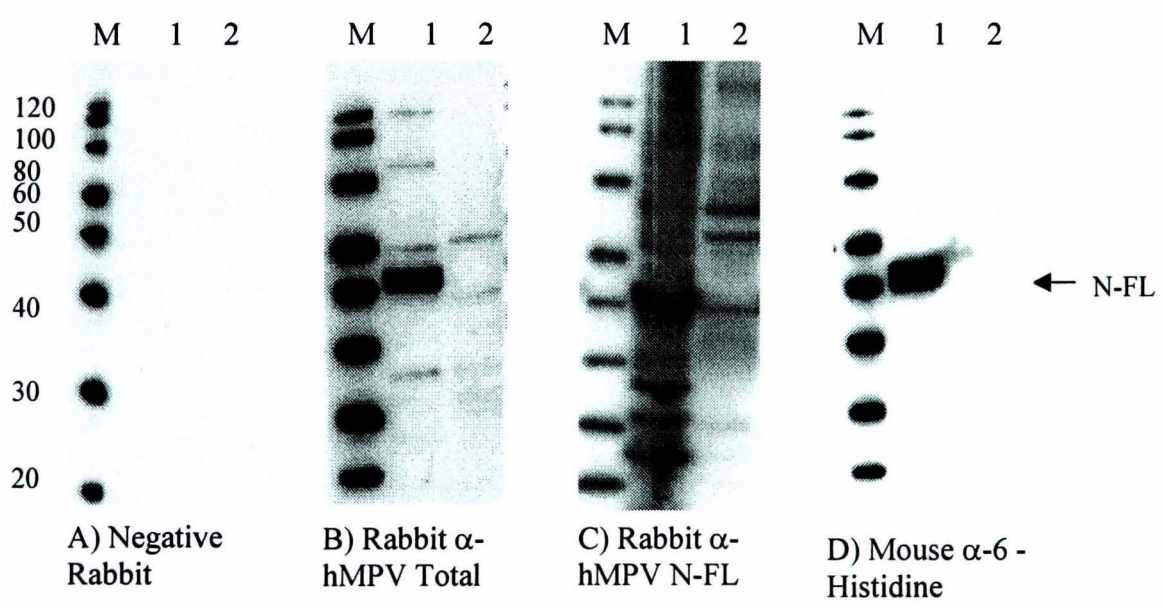


Figure 5.13: Western Blot analysis of hMPV specific Rabbit serum. (M) Marker, (1) Baculovirus hMPV N-FL infected SF9 cell lysate (2) Uninfected SF9 cell lysate (A) Negative rabbit serum 1:50, (B) Rabbit α-hMPV Total 1:50, (C) Rabbit α-hMPV N-FL 1:250, (D) mouse α-6-histidine MAb 1:1000. All rabbit sera were detected with goat α-rabbit IgG HRP conjugate at 1:2500 with ECL and autoradiography.

Table 5.6: Summary of molecular weight profile of the major species recognised by Mouse α-6-Histidine MAb, rabbit α- hMPV Total, rabbit α- hMPV N-FL, and negative rabbit serum by Western blot analysis.

Antigen	Mouse α-6-His	Rabbit α-hMPV Total	Rabbit α-hMPV N-FL	Negative Rabbit
N-FL cell lysate	51	51	51	Negative

5.6.3 Analysis of hMPV Specific Sera by Immunofluorescence against recombinant hMPV proteins

Sera were also tested against the recombinant baculovirus expressed hMPV proteins by indirect immunofluorescence of hMPV N-FL recombinant baculovirus infected/ uninfected SF9 cells grown on cover slips. Figure 5.14 shows the IF against baculovirus hMPV N-FL infected/ uninfected SF9 cells. Results show that both Rabbit α-hMPV N-FL and Rabbit α-hMPV Total react specifically against the N with no background binding to uninfected cells (Rabbit α-hMPV N-FL having been cross absorbed). As reflected in the ELISA results the Rabbit α-hMPV N-FL serum gave a greater level of fluorescence relative to that of the Rabbit α-hMPV Total as the Ab different dilutions tested here do not fully account for the difference in hMPV N antibody titre between the two sera demonstrated by ELISA, and therefore a lower dilution of Rabbit α-hMPV Total should be used for optimal fluorescence.

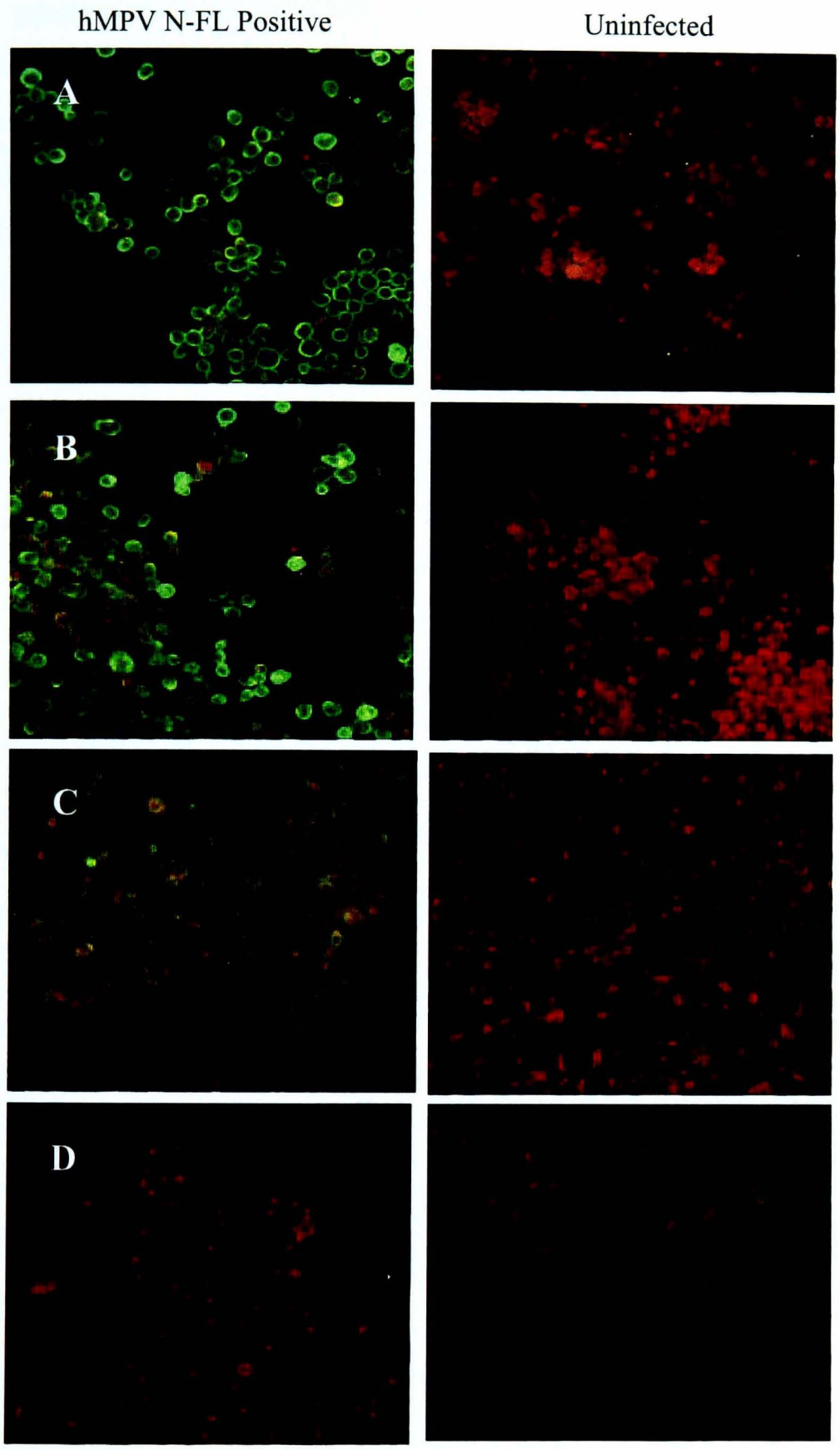


Figure 5.14: Indirect immunofluorescence analysis of hMPV specific antiserum against hMPV N-FL positive / negative SF9 cells. SF9 cells were grown on glass cover slips and infected with 3MOI of recombinant baculovirus hMPV N-FL. At 72 hours pi cells were acetone fixed. The rabbit serum was applied and detected using swine α-Rabbit IgG FITC conjugated Ab. Cells were counter stained with Evans Blue and visualised by fluorescence microscopy. (A) Mouse α-6-histidine 1:50, (B) Rabbit α-hMPV N-FL 1:25, (C) Rabbit α-hMPV Total 1:25, (D) Negative Rabbit Serum 1:25. Rabbit serum was detected with Swine α-Rabbit IgG FITC conjugate 1:40. Counter stained with Evans Blue.

5.6.4 Analysis of hMPV Specific Sera by Immunofluorescence against hMPV positive clinical isolate infected LLC-MK2 Cells

LLC-MK2 cells were grown on glass cover slips and inoculated with 250µl of hMPV infected LLC-MK2 passage 1 cell lysate material (unknown quantity of virus). The hMPV clinical isolates used were obtained from patients with ARTI from Northern Ireland (Kindly set to us from the Belfast HPA) 1 type A and 1 type B virus was used. At 14 days post infection cells were acetone fixed. Rabbit α-hMPV N-FL and rabbit α-hMPV Total was applied and detected using swine α-rabbit IgG FITC conjugated Ab. An FITC conjugated rabbit α-hMPV N-FL IgG was also evaluated for its use as a direct immunofluorescent agent. The conjugate was kindly prepared by Dr Dhan Samuels at the HPA Colindale. Cells were counter stained with Evans blue and visualised by fluorescence microscopy (Figure 5.15).

IF of hMPV infected/ uninfected cells showed that the rabbit α-hMPV Total antiserum recognised the hMPV infected LLC-MK2 cells while, unexpectedly the rabbit α-hMPV N-FL serum did not. There was however, a low level of fluorescence against hMPV subtype A with the rabbit α-hMPV N-FL FITC reagent as a direct IF reagent. There are a number of reasons that may explain the lack of reactivity by the α-N-FL antiserum; the first and most obvious being that there are conformational differences between the baculovirus expressed N-FL, against which the sera was raised, and the N-FL expressed in mammalian cells during infection. It may be that in natural infection the N is complexed with other viral or cellular proteins or indeed nucleic acid, which masks its recognition by the antibody. Alternatively the rabbit α-hMPV N-FL antiserum may be predominantly against the 6-histitine tag. This could be assessed by cleaving the Tag off and reanalysing sera reactivity.

With regard to the reactivity of the rabbit α-hMPV Total antiserum, this information in conjunction with the IF and ELISA data against the recombinant N-FL suggests that the majority of antibody in this serum is directed to hMPV proteins other than the N. Reactivity may also be affected by potential conformational differences between recombinant and natural N expression.

The results shown in Figure 5.15 were cells analysed at 14 days post infection. However analysis was also performed at 7 and 21 days post infection (data not shown). There was no difference observed between the time points.

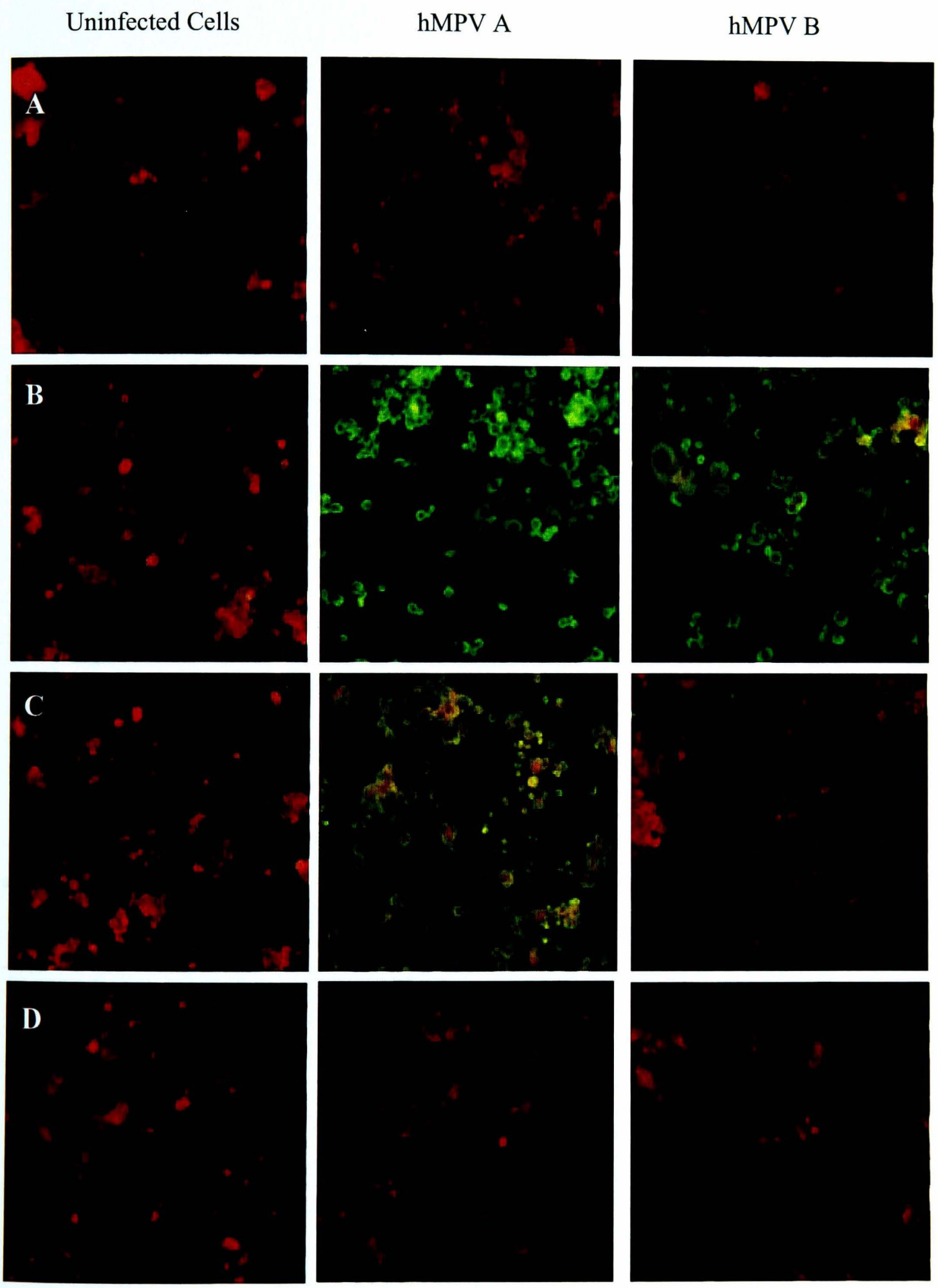


Figure 5.15: Indirect immunofluorescence analysis of hMPV specific antiserum against hMPV infected / uninfected LLC-MK2 cells (NI1-04 and NI2-04). (A) Rabbit α-hMPV N-FL 1:25, (B)

Rabbit α-hMPV Total 1:25, (C) Rabbit α-hMPV N-FL- FITC 1:25, (D) Negative Rabbit Serum. Rabbit serum was detected with Swine α-Rabbit IgG FITC conjugate 1:40. Counter stained with Evans Blue.

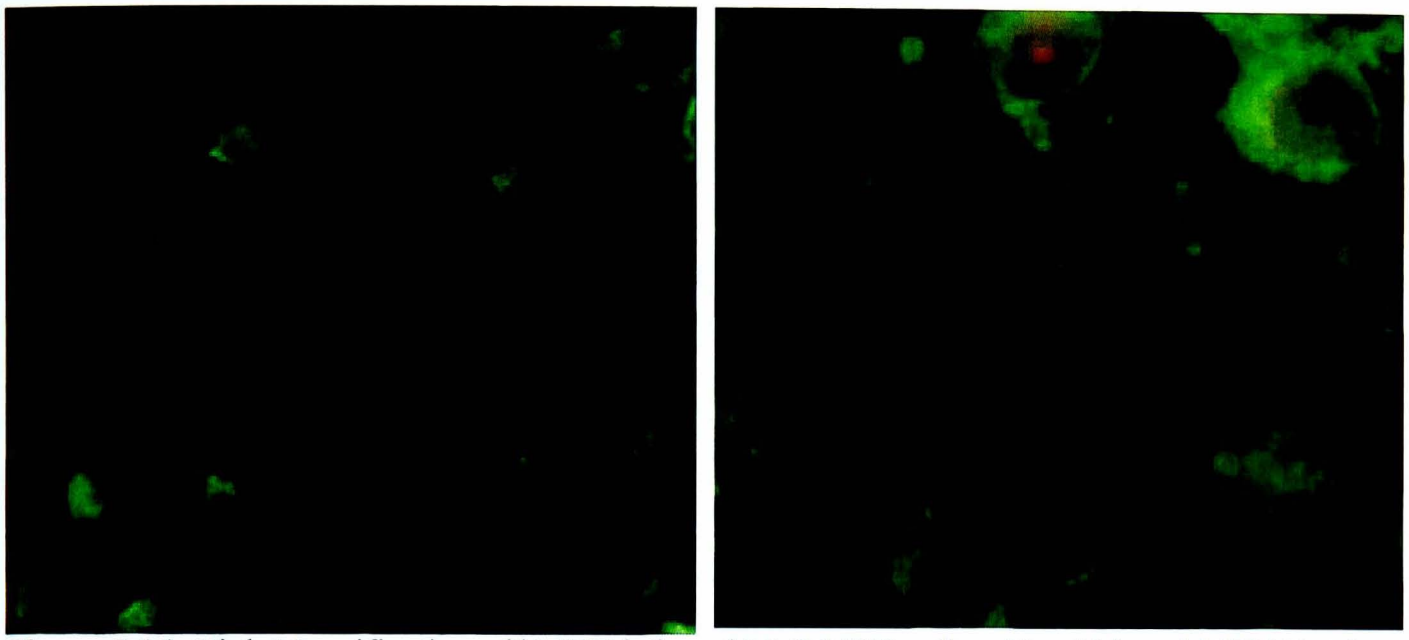


Figure 5.16: High Magnification of hMPV infected LLC-MK2 cells with rabbit α- hMPV Total.

Higher magnification images of hMPV infected LLC-MK2 cells (Figure 5.16) immuno-stained with rabbit α-hMPV Total show that the viral proteins were cytoplasmically located. The cells have a speckled appearance, which is slightly more concentrated around the nuclear envelope.

5.6.5 Analysis of Rabbit α-hMPV N-FL against N-FL expressed in mammalian cells

To investigate further the reactivity of the rabbit α-hMPV N-FL antiserum against N-FL expressed in mammalian cells, human kidney cells (293T) were transiently transfected with the hMPV N-FL pTriEx plasmid. Expression of N-FL was analysed by immunofluorescence and western blot. For immunofluorescence cells were fixed with 80% acetone PBS or 4% paraformaldehyde and permeablised using 0.25% TritonX-100 at 24 hours post transfection. Surprisingly immunofluorescence analysis of the transfected cells with the mouse α-6 histidine MAb, rabbit α-hMPV N-FL, rabbit α-hMPV Total, and a range of adult human serum known to be seropositive for hMPV N all yielded negative fluorescence results (Data not shown). By Western blot analysis however the 51KDa hMPV N-FL was detected, by the mouse α-6 histidine MAb, rabbit α-hMPV N-FL, and rabbit α- hMPV Total antiserum (Figure 5.17) confirming the expression of the N-FL and confirming recognition of linear epitopes by these sera. The α- N-Fl serum however also recognises a doublet at approximately 70KDa, which is also seen in the negative serum.

The α- hMPV Total serum also recognises a number of other proteins which are not detected by the α-6-histidine or other rabbit serum. These bands are present in both the infected and uninfected cell lysate. Therefore these non-specific bands are likely to be due to cross reactivity with cellular proteins. The 51KDa band size of the N-FL recognised here is the same size as that expressed in the recombinant baculovirus system which suggests that the post translational modifications are similar in both cell types.

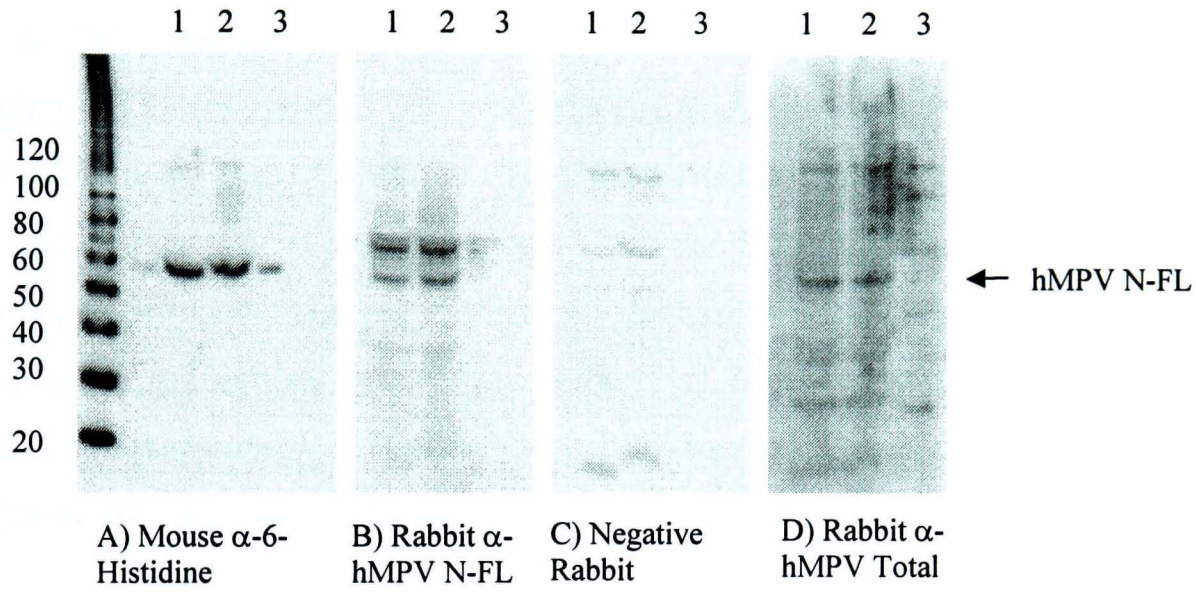


Figure 5.17: Western blot analysis of hMPV N-FL transfected 293T cell lysate 24 hours post transfection. (A) Mouse α-6 histidine MAb 1:1000 detected with Rabbit α-Mouse IgG HRP 1:10000, (B) Rabbit α-hMPV N 1:500, (C) Negative Rabbit 1:50, (D) Rabbit α-hMPV Total 1:50. A, B and C were detected with Goat anti Rabbit IgG HRP at 1:2500. Against (1) hMPV N-FL transfected 293T cell lysate 1, (2) hMPV N-FL transfected 293T cell lysate 2, (3) 293T un-transfected cell lysate.

These data support conclusions that there may be differences between the baculovirus expressed N and that expressed in mammalian cells through transfection or hMPV infection. Detection of the N-FL by Western blot of the transfected 293T cell lysate however indicates linear epitopes are recognised by the rabbit α-hMPV N-FL serum. It may be that the levels of N in the hMPV infected cell lysate/ transfected cells were too low for detection by IF. The fixation methods used for the detection of hMPV N-FL may also affect whether the protein is detected or no. When IF was performed in SF9 cells, cells were fixed with 80% ice-cold acetone, and worked well. This method was also used against hMPV infected and transfected mammalian cells, however results were negative for the α-N-FL rabbit serum. 4% paraformaldehyde was also tried on transfected cells followed by permeablisation with 0.25% Triton X-100 but again the results were negative.

5.6.6 Analysis of hMPV specific serum against hMPV infected and uninfected cell lysate and hMPV N expressed in the recombinant baculovirus system.

To further assess the reactivity of the hMPV specific antiserum, they were tested by Western blot against 2 different hMPV strains isolated from the 2 Northern Ireland clinical samples used in Figure 5.15, and purified hMPV N-FL were analysed (Figure5.18).

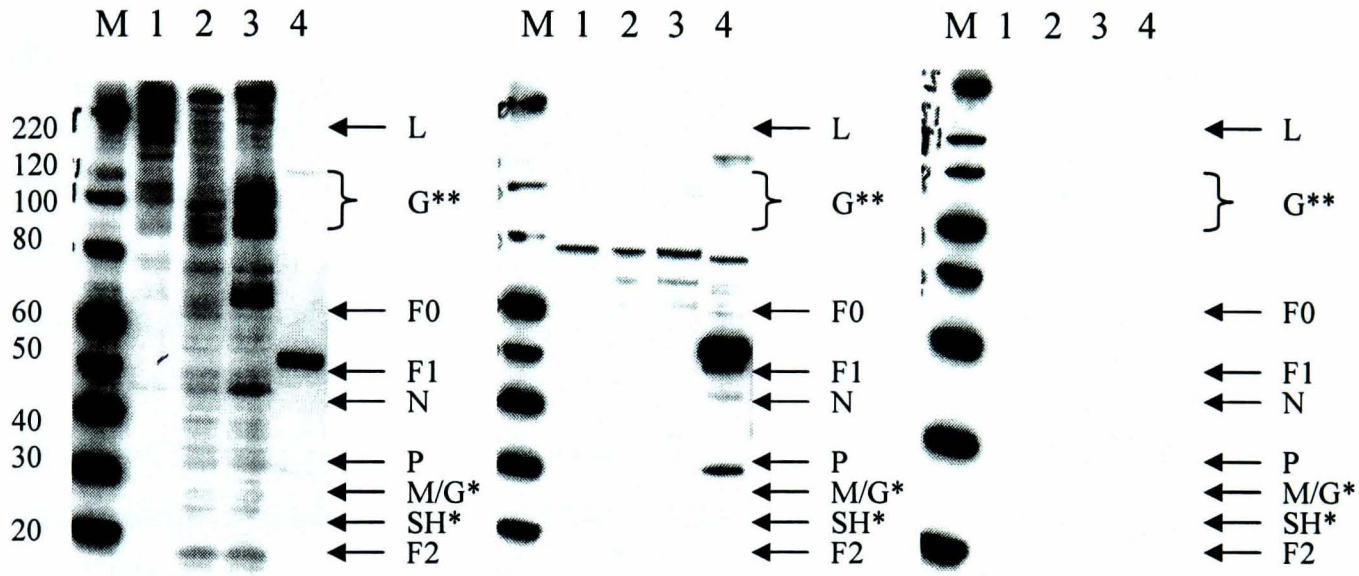
Panel A shows the results for rabbit α-hMPV Total. As shown previously this serum recognises the 51KDa band of the baculovirus expressed N-FL in lane 4. In this lane there is also a faint band at approximately 100-110KDa which may represent a dimeric form of N-FL, despite the use of denaturing gels. This band was also detected by the α-N-FL serum (Panel B lane 4); however, this was not detected by the α-6-histidine MAb (Data not shown) and therefore may just be an artefact. To determine if this is an N dimerised product a non-denaturing gel should also be run in parallel to confirm this result, as this gel system may therefore not be completely denaturing.

Against the hMPV infected and uninfected LLC-MK2 cell lysate The rabbit α-hMPV Total (Panel A lanes 1-3) there were a number of bands recognised, which were more apparent in lane 3 that were not detected in the uninfected cell lysate in lane 1. This included a band at molecular weight of 43-45KDa, which was the correct size for either the native nucleocapsid protein or the fusion protein F1 fragment. There was also a band at 90KDa recognised in lanes 2 and 3 by the rabbit α-N-FL in panel B, and supports possible recognition of an N dimer. There are also 2 bands of approximately 90 and 100KDa in lanes 2 and 3, which may be glycosylated species of G. These bands differ in the intensity in which they are stained (weaker in lane 2), and size, with those in lane 3 having slightly larger molecular weights than those in lane 2. This may demonstrates possible size or glycosylation differences between the G proteins of the two different strains analysed.

The rabbit α-hMPV Total (Panel A) also recognises 2 another bands in lanes 2 and 3, the first with the approximate molecular weight of 60-65KDa which is the correct size for the native un-cleaved precursor fusion protein, and the second is the lowest molecular weight band detected which may represent the fusion protein F2 fragment neither of these bands are recognised by any of the other serum, as expected. Bands are present in lanes 2 and 3 which also correspond to the correct molecular weight for the P and M proteins.

Panel C is negative rabbit sera and as expected shows no or very little reactivity against all antigens.

This analysis highlights the need for monoclonal antibodies against each of the hMPV proteins in order to confirm the reactivity of the antiserum. The analysis of different cellular fractions and the use of non-denaturing gels will also aid in addressing or confirming these findings.



A) Rabbit α-hMPV Total **B) Rabbit α-hMPV N-FL** **C) Negative Rabbit**

Figure 5.18: Western blot analysis of various hMPV specific serum against recombinant hMPV proteins and hMPV infected cell lysate. Against (1) Uninfected LLC-MK2 Cell Lysate, (2) hMPV Cell Lysate –NI1-04, (3) hMPV Cell Lysate –NI2-04, (4) Purified recombinant N-FL. Table 5.2 shows the expected hMPV protein sizes * un-glycosylated, **glycosylated.

5.7 Discussion

Reported here is the use of the recombinant baculovirus system to express the hMPV nucleocapsid and the subsequent generation and evaluation of hMPV specific antiserum; required for the development of serological assays to further the investigation of hMPV.

Recombinant baculovirus expression of hMPV N-FL and N fragments

The results demonstrate that the hMPV N-FL could be expressed efficiently in the recombinant baculovirus system. The expression of the N fragments, however, was not as straight forward. The recombinant baculoviruses expressing N fragments A-E grew less efficiently in cell culture producing viral titres 2 logs lower than that of N-FL. Despite attempts at optimising the conditions, the level of expression was also considerably lower.

There are a number of reasons that may explain the low level of recombinant virus growth and or protein expression including the accumulation of defective virus particles. However, it is unlikely that this is the cause of the problem with these recombinant viruses, as the appropriate measures (use of the correct MOI of virus, and use of low passage number virus stocks) were used. Furthermore, newly constructed batches of these recombinant viruses also failed to propagate and express the N protein fragments at sufficient levels.

The level of protein expression was different for each of the fragments, with fragments A and D being expressed in slightly greater quantities than the others. When analysed by Western blot, 2 bands of the expected size of the monomeric and potentially dimeric forms of protein fragments A and D were detected, while only monomeric forms were detected for fragments B, C and E. This may be evidence of instability of these truncated proteins, which enhances the rate degradation. Fragments A and D, therefore, may be more stable as they may have the ability to dimerise, hence the greater level of protein detected.

There is evidence that multimeric/ dimeric forms of the N proteins of the *Paramyxoviridae* exist through the observation that the nucleocapsid proteins self assemble into capsid like structures when expressed in recombinant systems (Bhella et al., 2002, Fooks et al., 1993, Meric et al., 1994), and the domains involved in N-N assembly have been mapped to the N terminus (Krishnamurthy & Samal, 1998, Nishio et al., 1999). The presence of N dimers

may be a significant step in the assembly of the nucleocapsid. Non-denaturing protein gels, analytical ultra centrifugation sedimentation or size exclusion chromatography could be used to determine the presence of dimeric forms. The fact the potentially dimerised forms of the protein fragments were detected indicates that the denaturing gel system used in this study may not have been completely denaturing, and the addition of antioxidants to the running buffer may be required.

To investigate whether instability is the cause of the low level of the N fragment expression the relative quantity of heterologous gene mRNA and protein expression could be determined by northern and Western blot analysis respectively and compared with that of other baculovirus proteins expressed under the same promoter. The level of protein expression relative to the mRNA can therefore be determined. The mechanisms involved in the targeting of proteins for proteolytic degradation in insect cells are yet to be determined. However, in other eukaryotic cells unbiquitination plays an important role in this function and therefore ubiquitation inhibitors could also be examined to see if protein stability is improved (Wilkinson, 2000). It has also been suggested that a homologue for cystine protease; V-cath, is expressed during the late phases of baculovirus infection causing protein degradation (Slack et al., 1995), and that the presence of cysteine protease inhibitors could help to stabilise the expressed protein.

The N fragments in their truncated form may also have a toxic effect on the cells resulting in decreased virus propagation and protein expression levels. Cellular toxicity of heterologous proteins has been documented previously in this system.

This may be overcome by expression using the in vitro coupled transcription translation expression system as an alternative method for protein production and to help address the question of protein toxicity, as heterologous protein expression is not dependent on the growth of a virus and expression of the heterologous protein is not affected by the presence of other viral proteins.

Problems associated with the solubility of the truncated proteins may also be involved and could be overcome by fusing the N fragments to a fusion protein such as the maltose binding protein or IgG Fc protein which have been shown to be useful for increasing protein solubility in recombinant systems (Sachdev & Chirgwin, 2000, Sugiura, 2003).

Purification of hMPV N-FL

Purification of N-FL was required for the generation of α-hMPV N-FL specific antiserum. The purification system used was a Ni-NTA resin system which took advantage of the 6-histidine tag fused to the N-terminus of N-FL. Optimisation of the imidazole concentrations in the buffers used reduced the amount of contaminating proteins in the purified protein preparation. However, analysis of the preparation by densitometry indicated the presence of contaminating proteins, furthermore, the antiserum generated from this preparation cross-reacted with cellular proteins in Western blot analysis. This background reactivity was removed through cross-absorption. However, improvements of the protein purification techniques would be more efficient in the long term.

Two-stage protein purification would be likely to give the best results. By taking advantage of the inherent properties of the N as a nucleic acid binding protein heparin affinity columns may be used for the first round of purification. Due to the poly anionic nature and structure of heparin it is able to mimic the overall binding properties of nucleic acid, and has been used for the purification or evaluation of many other proteins (Karger et al., 2001, Tseng et al., 1999). Binding of hMPV N-FL to heparin has been demonstrated (data not shown), and provides additional evidence that the integrity of the N-FL was maintained. This first stage purification could then be followed by the Ni-NTA affinity chromatography as described previously to remove other contaminating nucleic acid, and heparin binding proteins which may be present. However, this 2-step approach is likely to result in a reduced yield of the purified protein.

It was important to ensure the integrity of the N-FL was maintained in order for the antigen to be used efficiently for the development of serological techniques, and the generation of antiserum. Binding of N-FL to heparin indicated that N-FL has maintained its nucleic acid binding function. Furthermore, the baculovirus expressed N-FL protein was recognised by the rabbit α-hMPV total antiserum in Western blot, IF and ELISA. However reactivity against N-FL was only week, whilst there was strong reactivity against hMPV infected cells. This may be as a result of low levels of hMPV N antibody in the serum, or more likely antigenic differences between native and baculovirus expressed N protein, or a combination of both. Therefore conclusions cannot be drawn as to whether antigenicity of the baculovirus expressed N-FL protein was properly maintained. As mentioned above

previous work by Méric *et al* has shown that the N protein of hRSV expressed in the baculovirus system forms nucleocapsid like structures, similar to those seen in RSV infected cells (Meric et al., 1994). If more time was available analysis of SF9 infected cells expressing hMPV N-FL by thin section electron microscopy would have been used to ascertain if this was also the case for hMPV N-FL.

At the later stages of baculovirus infection an N-FL doublet (50 and 51KDa) was visible by Western blot analysis. Although this was only observed in later time points, it does not exclude the possibility that doublets were present throughout, but were undistinguishable due to the quantity of antigen and exposure used. This may represent differences in the level of post-translational modification, the most likely being phosphorylation, although this was not investigated within the scope of this work. The use of metabolic labelling with P^{32} over a time course, and phosphorylation inhibitors in conjunction with Western blotting and mass spectrometry could be used to investigate if the N-FL is phosphorylated, to what extent, and whether the doublet is a result of a decline in phosphorylation.

Alternatively this could be a cleavage product. It has been reported that the nucleocapsid proteins of many RNA viruses, including influenza and SV5 are cleaved by proteases present in the cell (Mountcastle et al., 1974, Zhirnov & Bukrinskaya, 1981, Zhirnov et al., 1999). Ultimately analysis of the amino acid sequence of both species would address the question of whether the protein is cleaved or truncated versus differences in post translational modifications, and indicate where truncations may have been made before the use of protease inhibitors is investigated. Analysis of mRNA species by northern blot would also indicate whether the fragment is generated prior to translation. Furthermore, the expression of N-FL in a prokaryotic or in-vitro system could also be used to generate non-post translationally modified species for comparison.

HMPV Specific antiserum

High titred antiserum specific for hMPV is an essential tool for hMPV research. This chapter outlines the generation of hMPV specific antiserum: Rabbit α-hMPV total; rabbit α-hMPV N-FL and evaluates their reactivity against hMPV antigens in different assays.

Rabbit α-hMPV Total was raised against whole hMPV virus cell lysate. As expected this antiserum recognises hMPV in infected cells by indirect IF, and as such would be a useful tool for monitoring the growth of this virus in cell culture. Although not evaluated here it may also be useful as a diagnostic IF reagent used directly on clinical samples.

		Anti-serum			
Antigen Preparation	**Analysis**	**Mouse α-6-Histidine MAb**	**Rabbit α-hMPV N-FL**	**Rabbit α-hMPV Total**	**Rabbit α-hMPV N-FL FITC**
Recombinant baculovirus hMPV N-FL	**ELISA**	+++ (1:1000)	+++ (1:50)	+ (1:50)	ND
	WB	+++ (1:1000)	+++ (1:500)	++ (1:50)	ND
	IF	+++ (1:100)	+++ (1:50)	++ (1:25)	+ (1:20)
hMPV infected cells	**WB**	ND	- (1:100)	++ (1:50)*	ND
	IF	ND	- (1:25)	+++ (1:25)*	+/- (1:20)
Transfected 293T cells	**WB**	+ (1:1000)	+ (1:100)	+ (1:50)	ND
	IF	- (1:50)	- (1:50)	- (1:25)	ND

Table 5.7: Summary of hMPV specific antiserum reactivity against recombinant hMPV N-FL and hMPV infected cells/ cell lysate.

Foot notes: WB: Western blot analysis; IF: Immunofluorescence, (-) No antibody reactivity (+) Weak antibody reactivity, (++) moderate antibody reactivity, (+++) strong antibody reactivity. The values given in (brackets) represent the antibody dilution which was tested or yielded a positive result. (*) See also chapter 6.

HMPV infected LLC-MK2 cells analysed with the rabbit α-hMPV Total antiserum by IF at high magnification showed distinctive signs of CPE; focal rounding of the cells, syncytium formation and cell destruction. Analysis was carried out over 3 time points of 7, 14, and 21 days pi however little difference was observed between them indicating that infection and CPE was not progressive. This may be due to the amount of inoculum used, which had an unknown quantity of virus. As 100% of the cells on the hMPV-infected slide were fluorescing it suggests that all the cells may have been infected upon inoculation or that the virus had spread to all the cells within the first 7 day time period. As CPE did not progress further it indicates the virus may have been inhibited in some way, perhaps by the

antiviral responses of the cell. Further investigation of hMPV virus growth should be carried out varying the quantity of inoculum, growth conditions and cell line. Recently Biacchesi *et al* (2005) have reported the recovery of hMPV from cDNA containing an enhanced green fluorescent protein, which has enabled improved virus yields to be obtained (Biacchesi et al., 2005). This would be the ultimate tool of choice for investigating further the growth properties of this virus.

The rabbit α-hMPV total antiserum also recognised a number of species in hMPV infected cell lysates analysed by Western blot. The species recognised correlate with the expected size of the N protein, a potential dimeric form of N, F0, F_1, P, M, and high molecular weight glycosylated species of G.

The α-hMPV total antiserum reacts specifically with the hMPV N-FL expressed in the baculovirus system by IF, ELISA and WB. The ELISA and IF data indicate that despite N specific reactivity the N Ab titre is relatively low. The very strong reactivity of this sera against whole virus therefore indicates only a small minority of Ab is targeted to the N, and a majority is against other hMPV proteins.

As expected the rabbit α-hMPV N-FL antiserum recognises the hMPV N-FL expressed in the baculovirus system by ELISA WB and IF. However, there is little evidence to suggest it recognises the native N in hMPV infected or hMPV N-FL PTriEx transfected cells when analysed by IF but this may be due to relative concentrations of the protein. Western blot analysis of N-FL transfected cells was however positive. This indicates that there may be differences in the sensitivity of the two assays or that the fixation methods used in the IF were not optimal and alternative methods should be investigated. It may also be a possibility that a majority of the rabbit α-hMPV N-FL antiserum may in fact be targeted to the histidine Tag. Analysis of the antiserum against the expressed protein with the tag removed would address this question.

In summary the hMPV N-FL has been successfully expressed in the recombinant baculovirus system, and purified for the production of hMPV N-FL specific antiserum which will be a useful tool for the development of hMPV N specific assays, however, further evaluation of its reactivity against native N is required. A rabbit α-hMPV Total antiserum was also evaluated for its reactivity against hMPV antigens, and has show to be

reactive against both the recombinant N-FL and other hMPV antigens and has proved itself a useful tool for immunofluorescence work.

Chapter 6

Development of an assay to detect human antibodies to hMPV N

6.1 Introduction

The assessment of the seroprevalence of hMPV in the UK population is important for determining the populations most at risk of infection, the age of primary infection, the persistence of maternal antibodies, and identifying those to be targeted for potential vaccines.

Serological findings indicate hMPV has been present in the human population for at least 50 years, and by the age of 5 years nearly all children have been infected (van den Hoogen et al., 2001). At the time of starting this work serological assays for hMPV consisted of immunofluorescence (IF) or ELISA against hMPV infected cell lysate (Boivin et al., 2002, Ebihara et al., 2004a, Ebihara et al., 2003, Falsey et al., 2003, van den Hoogen et al., 2001, Wolf et al., 2003). These assays however are dependent on successful growth of hMPV in the laboratory and as a result are often not suitable for large scale epidemiological studies, and the assays have not been well validated for potential cross-reactivity with hRSV. Furthermore, results may be inconsistent and subtype specific biases may exist to the different hMPV proteins.

Serological assays based on ELISAs have been developed for the detection of antibodies to APV, RSV and other members of the *Paramyxoviridae*. These assays have been developed using infected cell lysate but a number of assays based on recombinant proteins, including the matrix (M) and nucleocapsid protein (N) have also been developed and shown to be more sensitive and specific than whole virus approaches (Buraphacheep et al., 1997, Gulati et al., 2000, Gulati et al., 2001, Samal et al., 1993).

Of the hMPV proteins the N, M and F proteins have been shown to be the most conserved between the 2 lineages of hMPV (Bastien et al., 2003), which is in direct contrast with the G and SH proteins which are the most variable, and therefore better suited for the analysis of subtype or strain specific antibody responses. In 2001 a study showed that the N protein was better than M for the detection of antibodies to APV A, B and C (Gulati et al., 2001). Recently sero-cross reactivity between the N proteins of hMPV A and B lineages has been demonstrated, and it was concluded that N proteins from either A or B lineages could be used for the serological analysis of hMPV (Hamelin & Boivin, 2005).

The data presented in chapter 5 shows the successful expression of the hMPV N-FL protein in the recombinant baculovirus system. Recognition of this recombinant protein with hMPV specific polyclonal antiserum by a variety of methods suggests this may be a suitable antigen for the development of an hMPV specific ELISA.

Presented here is the use of the recombinant baculovirus expressed hMPV N-FL protein to develop a specific ELISA for the detection of human α-hMPV N antibodies, and the application of this ELISA to ascertain the seroprevalence of hMPV in the UK.

6.2 Aims

- Design and optimise an ELISA based on the recombinant hMPV N-FL for the detection of hMPV specific IgG.
- Apply the ELISA to clinical samples to assess the seroprevalence of hMPV in a panel of age-stratified serum from the UK.
- Use hMPV reactive sera identified in the ELISA to map human antibody reactivity to the N.

6.3 Clinical Studies

Table 6.1: Summary of human serum used in this study

Human serum	Source
Pooled human sera	Colindale HPA (2001) pooled adult sera negative for reactivity against influenza antigens submitted to the HPA for serological investigation of respiratory illness.
Human sera ≤ 15 years	Colindale HPA (2001-2004) sera submitted to the HPA for serological investigation of influenza.
Human sera ≥ 15 years	Colindale HPA (2001-2004) sera submitted to the HPA for serological investigation of influenza.
Adult acute and convalescent RSV A/B sera	Colindale HPA (2001) sera submitted as part of investigation in to the cause of ARTI in particular hRSV in community dwelling elderly > 65 years old (Chapter 3)
Paediatric acute and convalescent RSV A/B sera	Colindale HPA. Neonatal paediatric sera collected as part of a longitudinal cohort study of RSV infection in childhood (Althani, 2004).
Age stratified panel of sera ≤ 72 months	Colindale HPA. Sera from UK children submitted to the HPA for investigated of rash illness

6.4 HMPV N ELISA Development

In order to develop an ELISA that can be used to detect antibodies to the hMPV N in human sera, a basic indirect ELISA was developed. The time required for generating the quantity of purified N-FL required for ELISA development was not feasible for the timescale of this work, therefore, hMPV N-FL positive cell lysate was used and background reactivity taken into account by using hMPV N-FL negative cell lysate. The blocking and coating buffer conditions and antigen/ antibody dilutions were optimised using Rabbit α-hMPV N-FL, Rabbit α-hMPV Total, Negative Rabbit serum, and human sera. All optimisation steps were performed changing only one parameter at a time starting with the standard ELISA conditions outlined below. The final optimal conditions are summarised in Table 6.2.

6.4.1 Standardisation of the hMPV N-FL positive and negative cell lysate.

As cell lysate was used as the antigen for this ELISA it was important that any background reactivity that sera may have had against cellular proteins was taken in to account. Therefore both N-FL Positive and Negative cell lysates were used. The OD450 value generated from the N-FL negative cell lysate was subtracted from that of the positive to give the corrected OD450 value. However, to do this accurately, the amount of protein used in the N-FL positive and negative cell lysate was standardised such that the amount of total protein for the negative cell lysate was the same as the amount of total protein used for the N-FL positive cell lysate. The starting material was initially standardised by a cell equivalence of $15x10^6$ cells/ml in the lysate. A total protein assay based on the Bradford protein assay method was used to measure the total protein, and the negative cell lysate diluted to give the same total protein content as the positive cell lysate (Figure 6.1).

This data also shows that there is a total protein concentration of 0.8mg/ml of the cell lysate preparation. An attempt was made to determine the % of N-FL in the total cell lysate using coomassie stained protein gel, however due to the number of cellular proteins present the N-FL protein band could not be distinguished and therefore could not be quantitated.

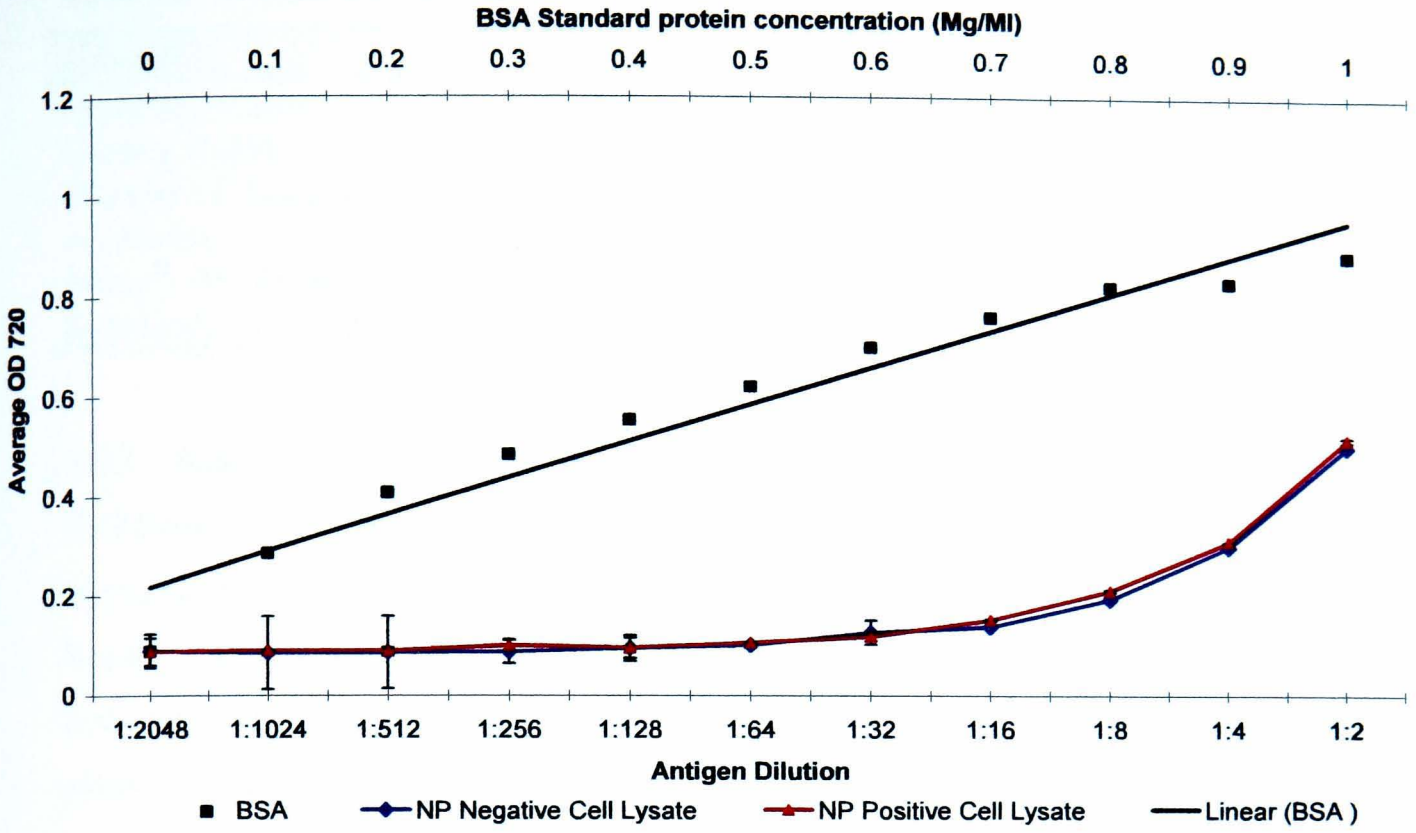


Figure 6.1: Total protein assay for the standardisation of the hMPV N positive and negative cell lysate. The Average OD720 values of the N-FL positive and negative SF9 cell lysate material in a 2-fold dilution series plotted against a standard BSA curve with a known amount of total protein. The error bars indicate +/- 1 standard deviation.

6.4.2 The standard ELISA conditions used as the basis for developing the hMPV N ELISA:

96 well U bottom plates were coated overnight at 4^0C with 100µl of hMPV N-FL positive or negative cell lysate diluted 1:100 in 10mM PBS pH7 to give approximately 0.8 µg of total protein/well. Plates were washed 4 times with 0.05% PBS Tween20 and incubated at 37^0C shaking at 600 rpm for 2 hours with 100µl of blocking reagent. Plates were washed and incubated as before for 1 hour with 100µl of primary antibody diluted 1:100 in 5% Milk in 0.05% PBS Tween20. Plates were again washed and incubated with 100µl of secondary HRP conjugated antibody diluted 1:2500 in 5% Milk in 0.05%PBST. Plates were washed for a final time and incubated in the dark with 100µl of TMB substrate for 5 minutes. The reaction was stopped with 100µl of 0.5M HCL. The absorbance at 450nm (reference 670nm) was measured using a 96 well plate reader. The negative OD value was subtracted from the N positive CL value to give the corrected OD

Table 6.2: Summary of optimised conditions used for the hMPV N ELISA

Condition	Options
Blocking Reagent	5% milk in PBS
Coating Buffer	20mM Tris-HCl pH 9
Dilution of Antigen for coating	1:200
Ab diluent	5% milk in 0.1% PBS – Tween 20
Primary Ab dilution	1:100
Secondary Ab dilution	1:1000

6.4.3 Determination of the optimal Blocking reagent

To determine the optimal blocking reagent control sera were tested against antigen coated/ uncoated plates blocked with either 1% BSA or 5% Milk in 10mM PBS pH7 (Figure 6.2). Results show the corrected OD450 values for each of the sera. When 1% BSA was used both positive control sera gave high OD readings in both antigen coated and uncoated plates. The addition of 1% BSA in the primary antibody diluent eliminated this, although not entirely, indicating the sera are reactive against BSA.

When 5% milk was used it gave good differentiation for both positive control sera between the antigen coated and uncoated plates, with no background binding in the uncoated plate. 5% milk in 10mM PBS pH7 was therefore chosen as a blocking reagent for this ELISA.

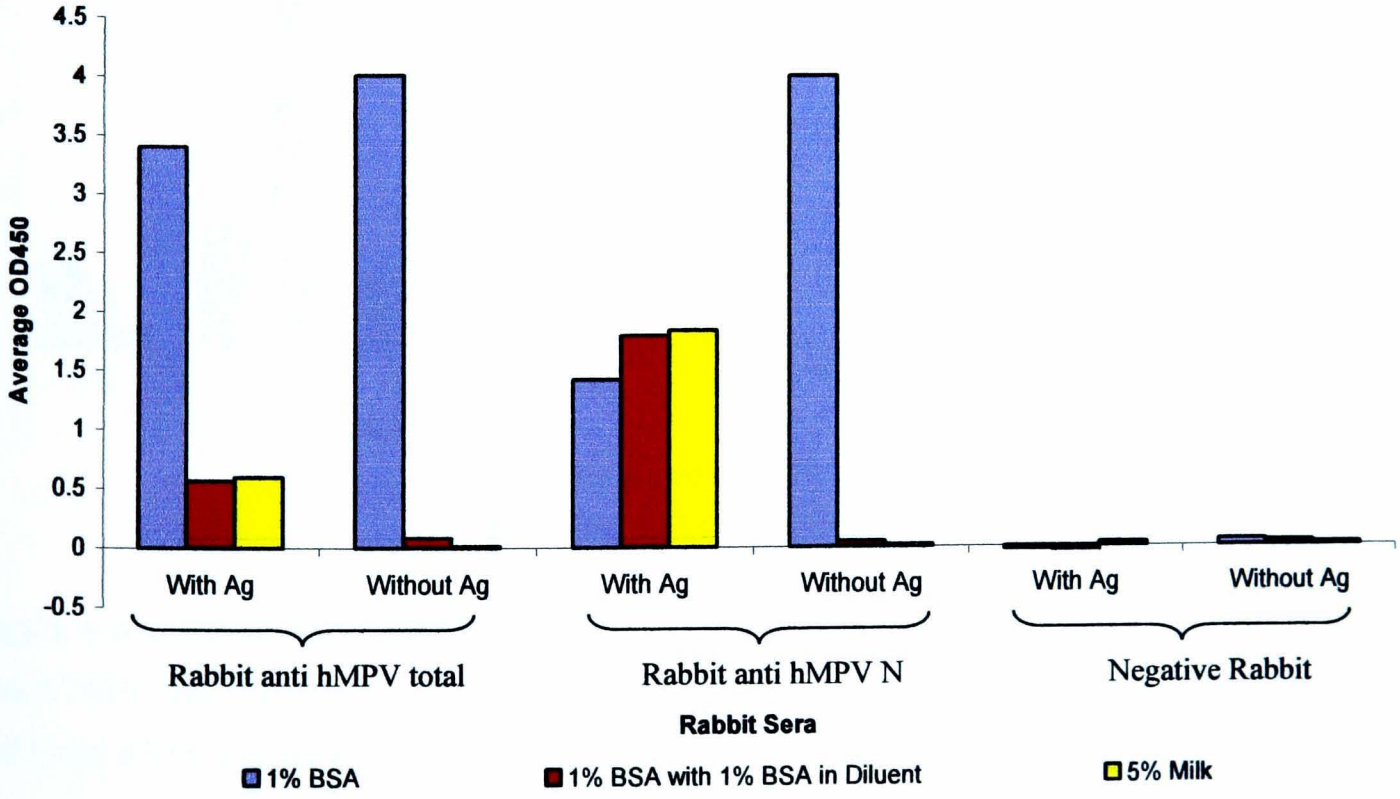


Figure 6.2: Blocking reagent optimisation. The corrected average OD450 values for Rabbit α-hMPV Total, Rabbit α-hMPV N-FL and negative Rabbit sera at 1:100 against Ag coated/ uncoated plates with 1% BSA, or 5%milk as a blocking reagent.

6.4.4 Coating Buffer

To determine the optimal coating buffer, plates were set up as described above with 20mM Tris-HCl pH 9, 10mM PBS pH7, or 10mM carbonate buffer pH9.6 as the coating buffer. Five % milk in PBS was used as the blocking reagent. A 10 fold dilution series of Rabbit α-hMPV Total, Rabbit α-hMPV N-FL, and negative Rabbit sera were tested (Figure 6.3). T/N ratios were calculated as the ratio of the OD450 values of the test sera divided by negative serum.

The results show that Tris- HCL at pH9 gave the highest T/N ratios for the Rabbit α-hMPV N-FL and Rabbit α-hMPV Total serum indicating the biggest difference between them and the Negative Rabbit sera. Tris–HCL was therefore selected as the optimal coating buffer for this ELISA.

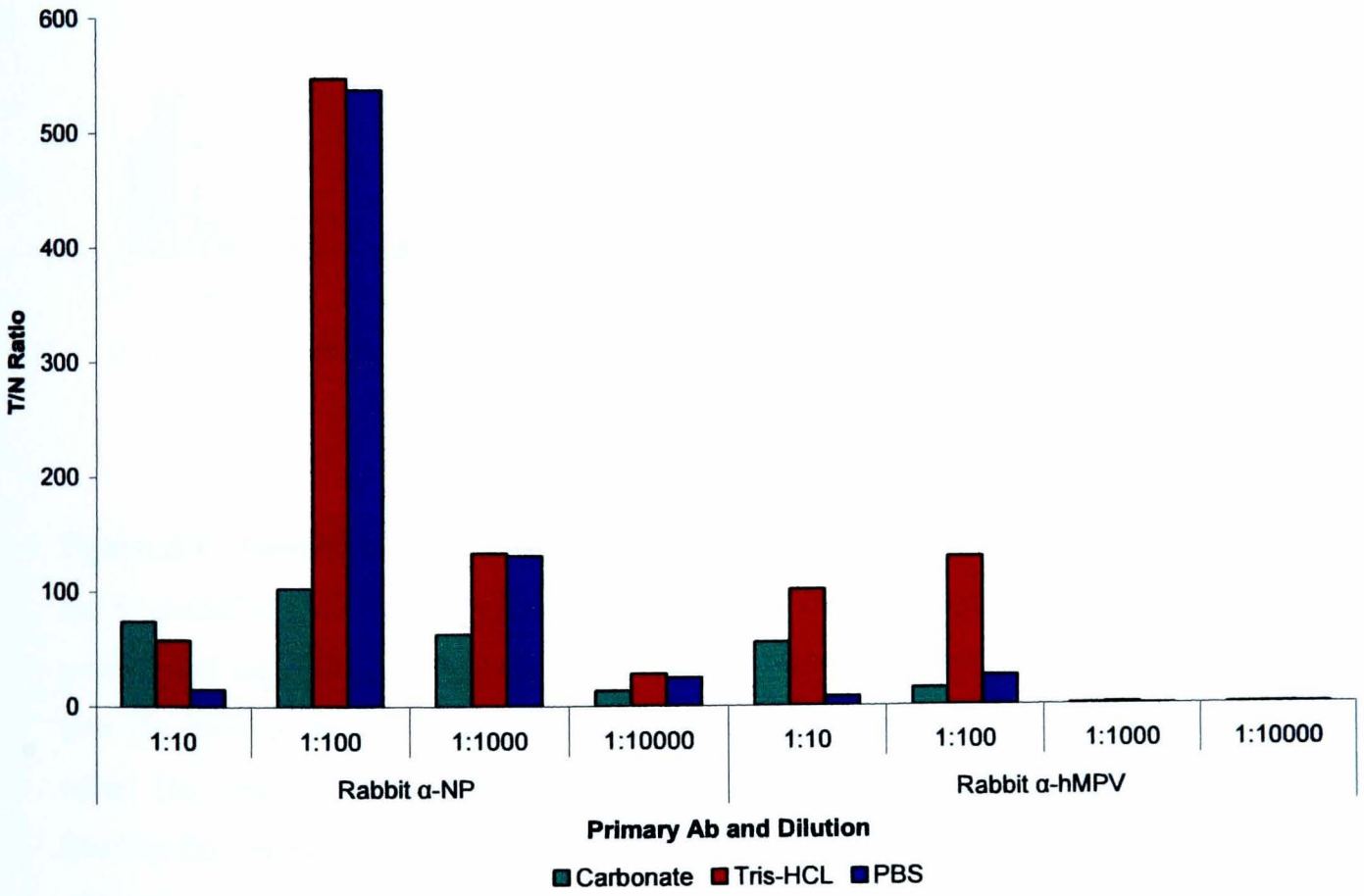


Figure 6.3: Coating Buffer optimisation. T/n ratio values for Rabbit α-hMPV Total, Rabbit α-hMPV N-FL sera in a 10 fold dilution series were tested against hMPV N-FL positive and negative cell lysate with Carbonate, Tris HCL or PBS coating buffers, repeated in triplicate.

6.4.5 Antigen dilution

To determine the optimal Ag dilution hMPV N-FL positive and negative SF9 cell lysates were diluted with Tris-HCL in a dilution series ranging from 1:50 to 1: 800. Plates were blocked with 5% Milk in PBS and tested with a dilution series of the rabbit control sera.

Shown below are the T/N ratios for Rabbit α-hMPV Total and Rabbit α-hMPV N-FL and sera in a dilution series (Figure 6.4). Results show that a 1:200 dilution of the Ag gives the optimal results at all Ab and Ab dilutions tested (Figure 6.4).

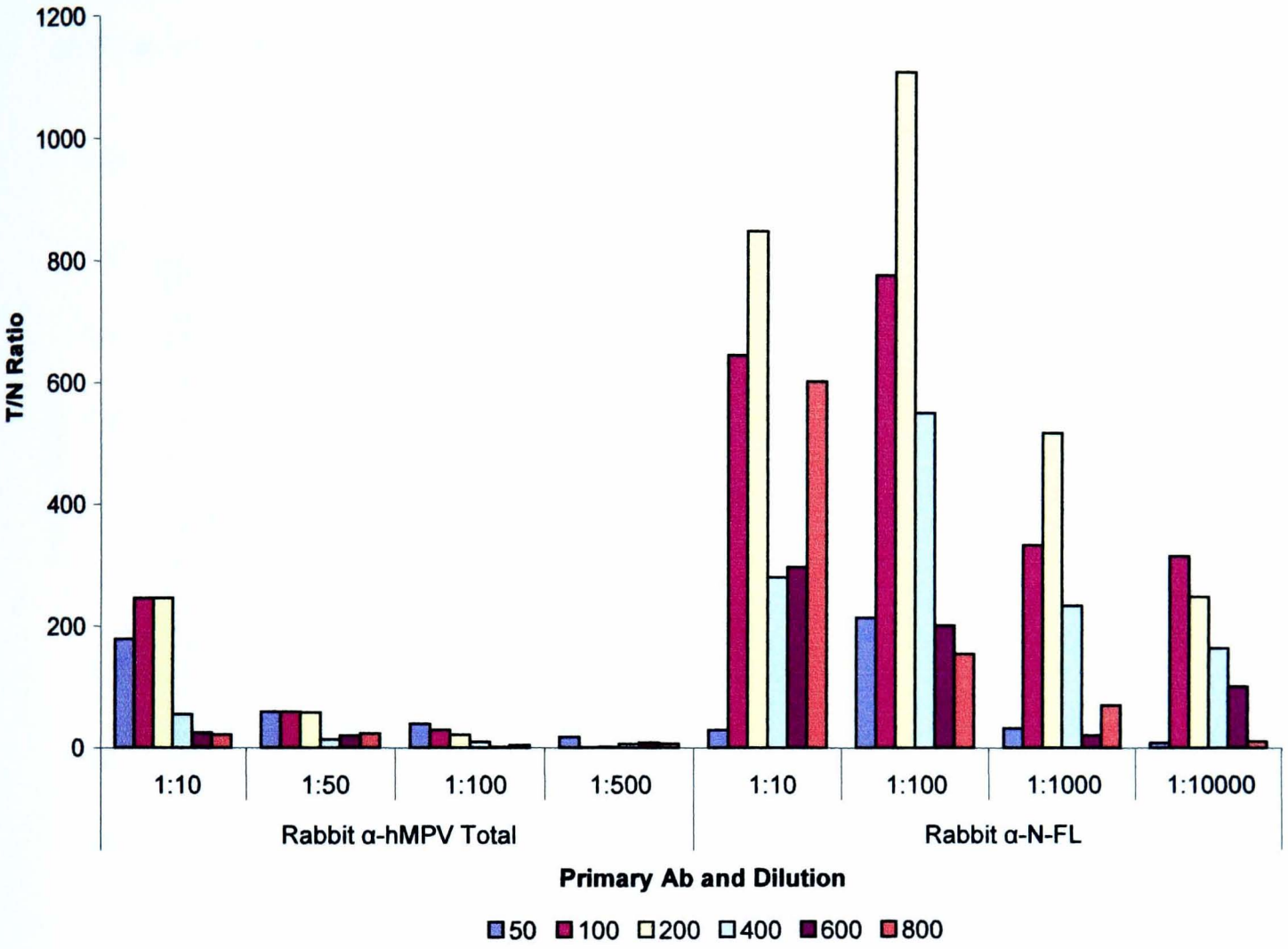


Figure 6.4: Antigen Concentration optimisation. The T/N ratios for Rabbit α-hMPV Total, Rabbit α-hMPV N-FL and negative Rabbit sera in a dilution series against a range of hMPV N-FL positive and negative cell lysate dilutions. The OD value for the negative cell lysate was subtracted from the positive for each replicate (each was tested in quadruplicate) to give the corrected OD value. The average corrected OD value was then determined. The T/N ration was calculated by dividing the corrected OD value for each test sera, by the corrected OD value for the negative rabbit sera.

6.4.6 Primary and secondary antibody dilution

Optimal primary and secondary Ab dilutions of the rabbit control sera were determined by testing a range of primary antibody dilutions with 1:1000 and 1:2500 dilutions of the Goat α-Rabbit HRP conjugated secondary antibody (according to the manufacturers suggested range) (Figure 6.5). Rabbit α-hMPV Total was optimal when used at 1:10 dilution, and is therefore a very low titre serum. However, a 1:50 dilution also gave an expectable OD

value, and clear results against purified N-FL by Western blot (Chapter 5). A 1:50 dilution of rabbit α-hMPV total was therefore chosen. Rabbit α-hMPV N-FL was optimal when used at a 1:100 dilution. The negative rabbit serum gave similar results at all dilutions tested and therefore also used at 1:100. The Goat α-Rabbit HRP conjugated secondary antibody was optimal at a 1:1000 dilution for all the primary rabbit serum dilutions tested.

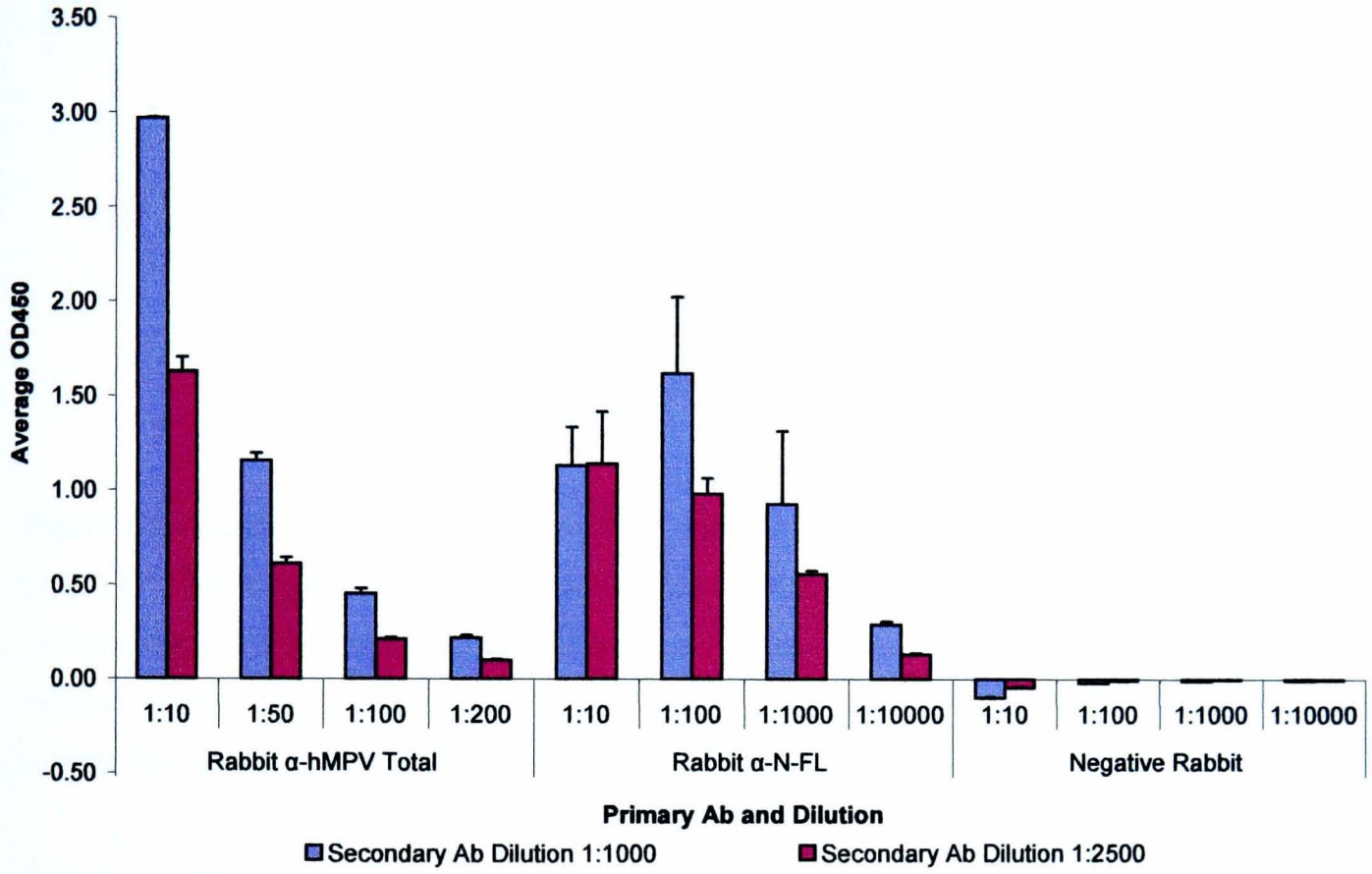


Figure 6.5: Control sera dilution optimisation. The corrected average OD450 values for Rabbit α-hMPV Total, Rabbit α-hMPV N-FL and negative Rabbit sera in a dilution series against the optimal hMPV N positive and negative cell lysate dilution. The OD value for the negative cell lysate was subtracted from the positive for each replicate (each was tested in quadruplicate) to give the corrected OD value. The average corrected OD value was then determined.

6.4.7 Human Ab reactivity in the hMPV N ELISA and establishment of a "Cut Off Value".

To determine whether human sera were reactive against the baculovirus N-FL in the ELISA, and if so determine the optimal dilution of sera to be used for routine testing and high throughput screening a panel of 5 different adult sera (expected to be positive for hMPV N IgG) were tested in a 10 fold dilution series and detected with Rabbit α-Human IgG HRP conjugated antibody at 1:5000 the manufacturers recommended dilution (Figure 6.6).

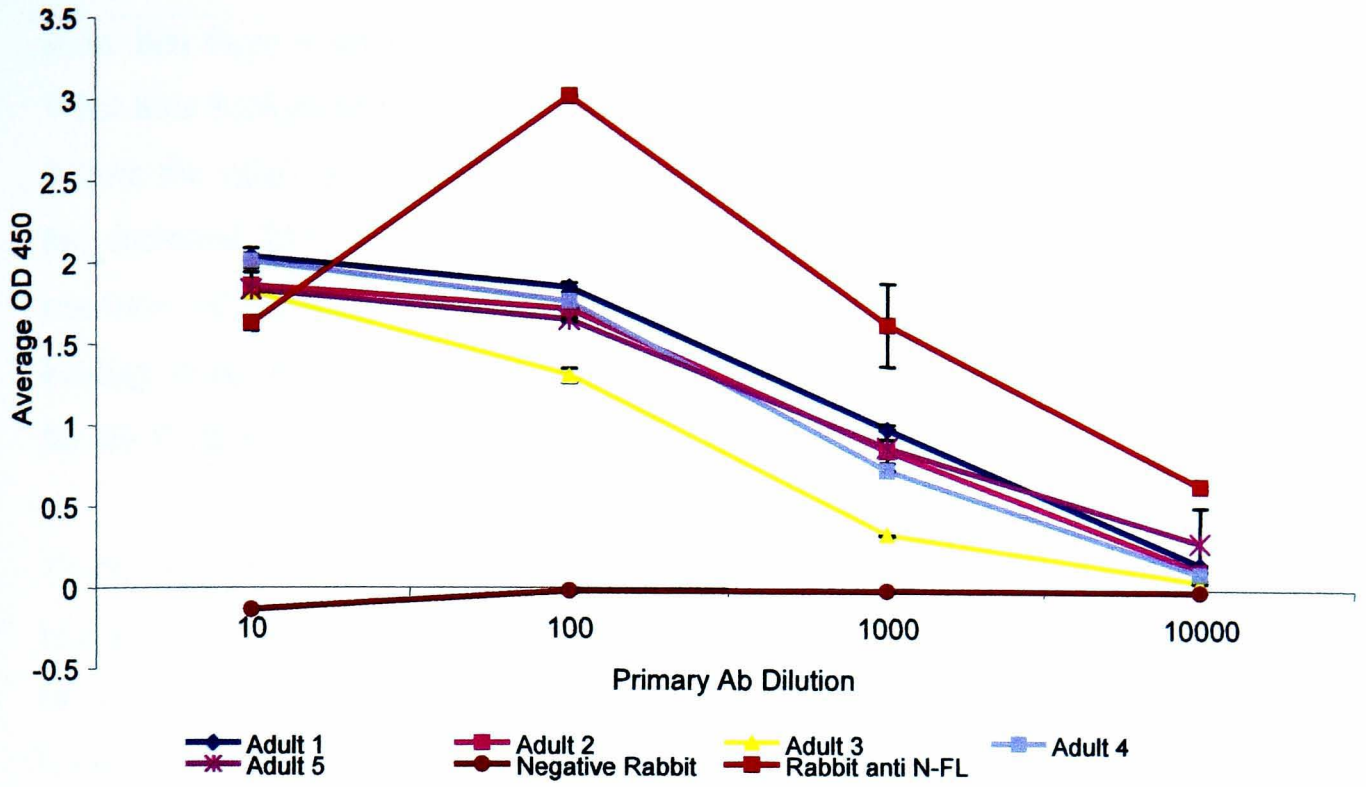


Figure 6.6: **Human Ab Reactivity and Dilution Optimisation**. The corrected average OD450 values for 5 adult human sera in a 10-fold dilution series against the optimal hMPV N-FL positive and negative cell lysate dilution. The OD value for the negative cell lysate was subtracted from the positive for each replicate (each was tested in quadruplicate) to give the corrected OD value. The average corrected OD value was then determined. Error bars show plus 1 Standard Deviation.

The results show the mean OD450 values for the sera at each dilution and indicate that all the adult sera tested were reactive against N-FL in this ELISA. The ideal dilution to be chosen should fall in to the linear part of the titration curve as this provides the greatest sensitivity. Therefore a dilution of 1:100 was chosen. This is a convenient dilution to use routinely as only a small amount of sample is required and it is unlikely that background interference will occur. It also gives a clear distinction between positive and negative reactive sera, as illustrated in Figure 6.7.

In order to establish the "Cut Off" for the assay, sera which are negative for hMPV N IgG needed to be identified. To do this serum from children aged 0-15 years that were submitted to the HPA Colindale for investigation in to Influenza like illness were screened in the hMPV N ELISA at a 1:100 dilution (Figure 6.7A).

Figure 6.7A displays the mean OD450 value of the human sera against both the N-FL positive (Blue) and N-FL negative (Red) cell lysates (Standardise total protein). The results

show that there is very little background binding of the sera to the negative cell lysate. What little background reactivity that does occur, however, is accounted for by subtracting it from the value obtained against the Positive cell lysate as described previously to give the corrected OD450 value. This is shown to be important for determining the true reactivity of the Rabbit α-hMPV N control sera, which has a high level of background binding to the N negative cell lysate. The horizontal black line represents the Cut Off value for this ELISA.

To determine the Cut Off value a number of low and moderately reactive sera, highlighted in Figure 6.7A by green and yellow arrows respectively, were also tested at a 1:75 dilution by Western Blot Analysis against purified N-FL (Figure 6.7B). The seven sera with the lowest reactivity by ELISA were also non reactive by Western Blot and were subsequently selected as negative controls to calculate the "Cut Off" value for the ELISA. The Cut Off value was set at an OD450 of 0.4, which was placed between the lowest OD450 value for the serum which was reactive by western blot, and the highest OD value for the sera which was negative by western blot (Figure 6.7).

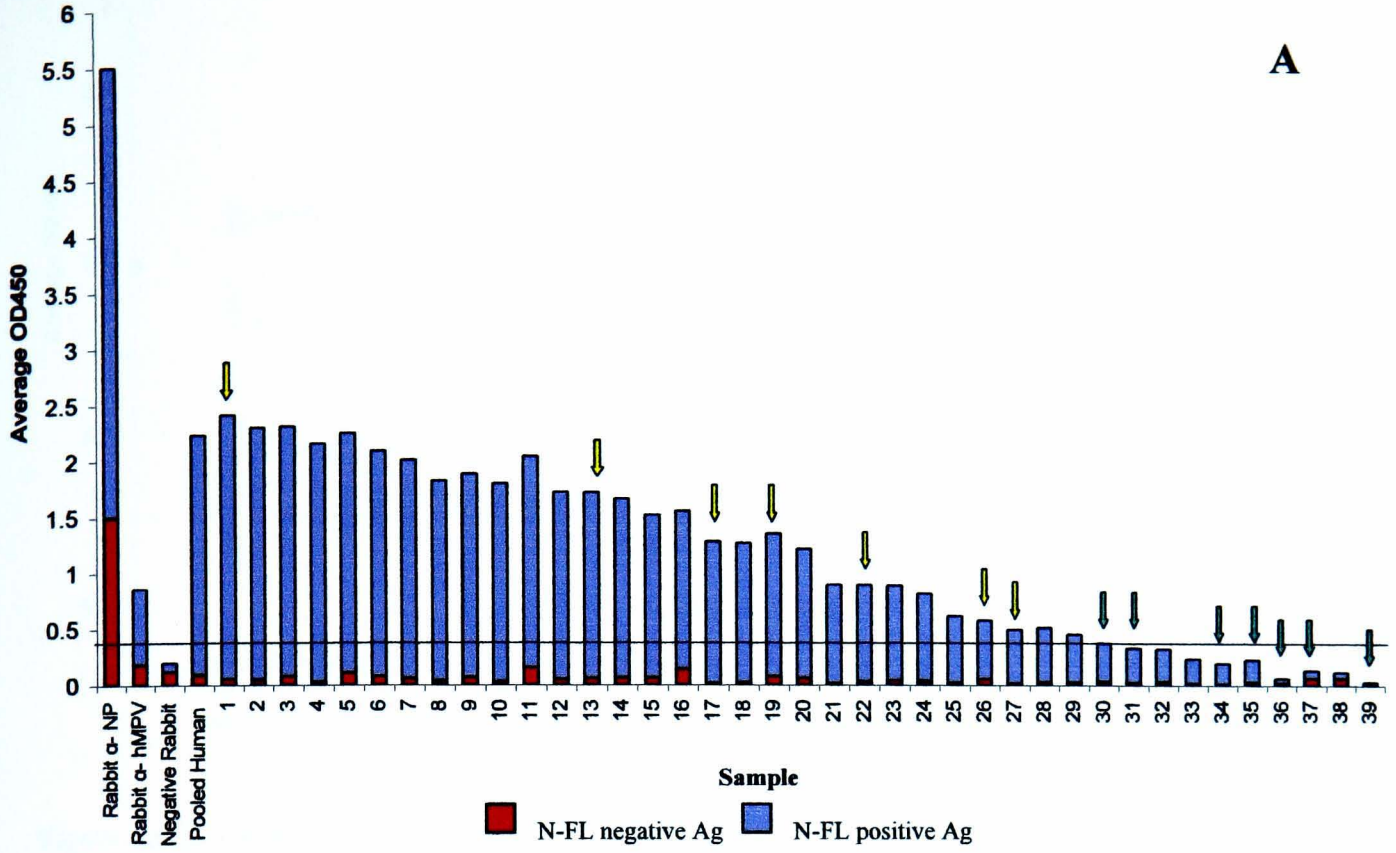


Figure 6.7: Identification of human negative serum and establishment of the cut off. (A) Shows the mean OD 450 values of paediatric sera reactivity against the N-FL positive and N-FL negative cell lysate, with the established cut off for the ELISA indicated. The samples highlighted by green and yellow arrows indicated the hMPV N-FL ELISA negative and positive sera analysed by western blot at a 1:75 dilution (B) against approximately 95ng of purified N -FL.

To ensure that sera were truly negative at lower dilutions with respect to the established "Cut Off" value, the same negative sera were also tested in a dilution series starting at 1:10 (Figure 6.8). Results show that all the sera were negative at all the dilutions tested with the exception of sample 36, which was weekly positive at 1:10, although at this low dilution it is more likely to be background interference than a genuine reaction against N-FL.

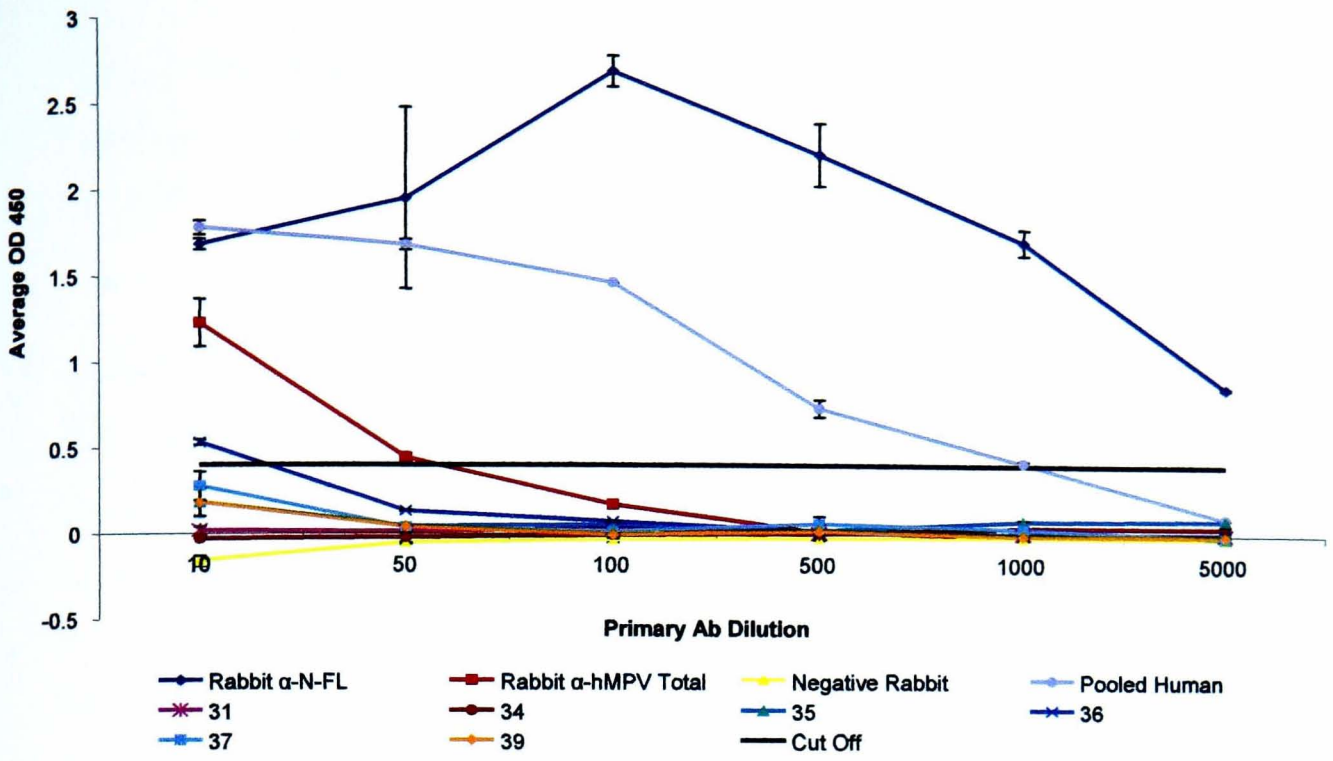


Figure 6.8: Conformation of Negative Human Serum. The corrected mean OD 450 values of paediatric sera in a dilution series with previously identified negative reactivity against N by ELISA at a 1:100 dilution and Western Blot analysis. The OD value for the negative cell lysate was subtracted from the positive for each replicate (each was tested in triplicate) to give the corrected OD value. The average corrected OD value was then determined. Error bars show plus 1 Standard Deviation.

6.4.8 hMPV N ELISA Specificity and analysis of sero- cross reactivity

It was important that this assay was specific for the detection of hMPV N IgG. Therefore to evaluate whether there was cross reactivity with antibodies against other common respiratory viruses, in particular hMPVs closest human relative hRSV, rabbit sera raised against a number of different respiratory viruses including RSV were tested in the hMPV N ELISA at 1:100 dilution (

Figure 6.9A). In addition, 22 pairs of acute and convalescent serum taken from infants during / following a primary RSV A or B infection were also tested at 1:100 in the hMPV N ELISA (

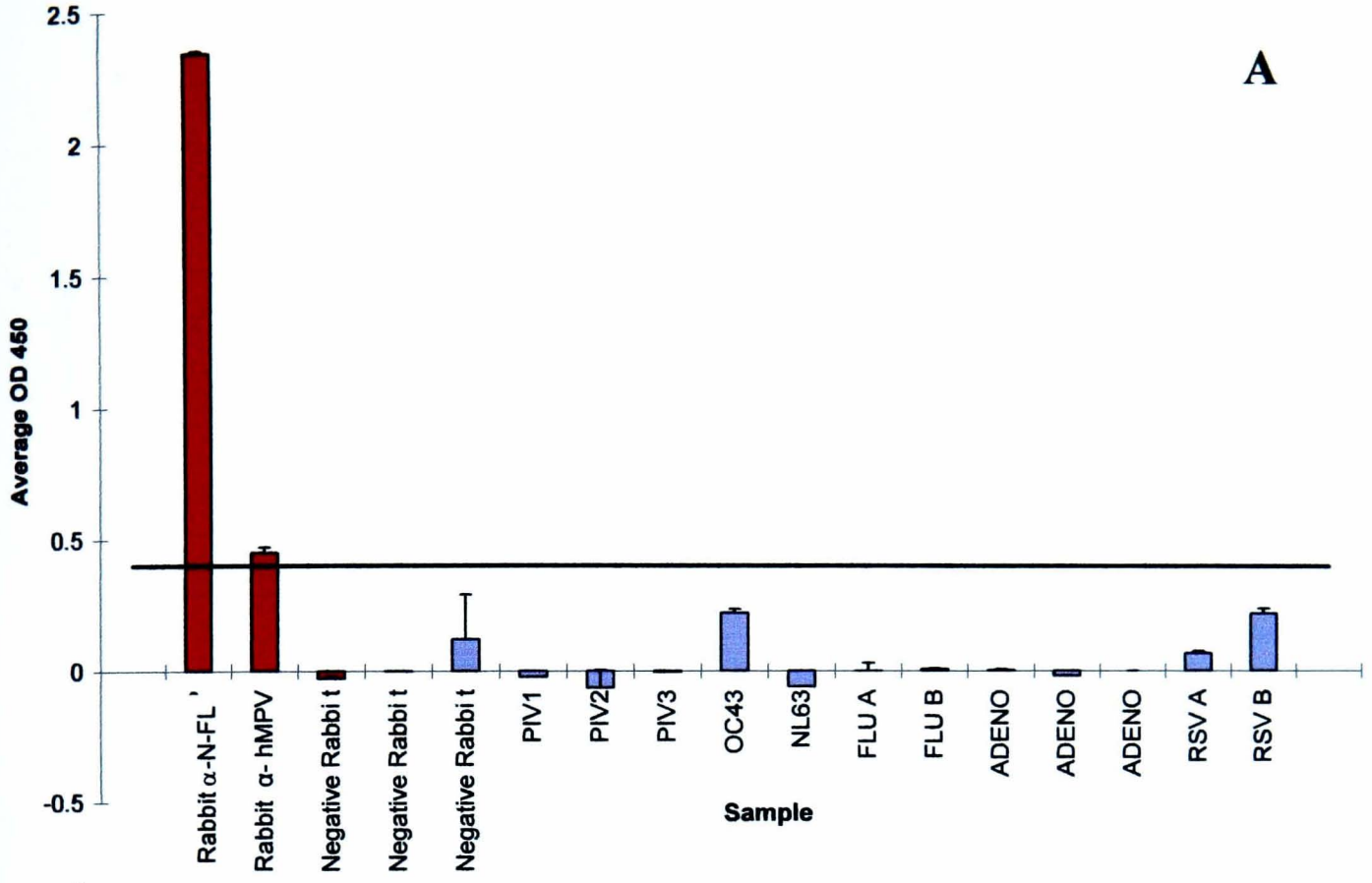
Figure 6.9B). Ideally to address this question fully the change in hMPV Ab titre between acute and convalescent RSV serum would be investigated, unfortunately due to the limited availability of the serum from these often very young children this was not possible.

Primary RSV infections were previously identified in these individuals using a combination of RT-PCR and RSV neutralisation assays (Althani, 2004).

Also, a panel of paired adult serum taken as part of the community dwelling elderly study described in chapter 3 were also analysed. These paired serum were taken following ARTI with hMPV, RSV, influenza or unknown aetiology and analysed at 1:100 (Figure 6.10 A-D).

As can be seen in
Figure **6.9**A all the rabbit sera tested that were raised against other common respiratory viruses fell below the Cut Off value for the ELISA. Furthermore
Figure **6.9**B shows there was no increase in Ab reactivity observed when 22 pairs of acute and convalescent paediatric sera following primary hRSV A/ B infections where tested in the ELISA. On average there was in fact a decrease in the OD450 value in the convalescent serum, perhaps representing a decrease in maternal hMPV N antibody titre.

In addition to this Figure 6.10 A-D shows the corrected mean OD450 value for the individual acute and convalescent serum following hMPV, RSV, influenza or other infection, with the average values for each cohort. The results show that all the adult sera were seropositive for hMPV, as expected, however, only the hMPV data set showed an increase in OD450 values in the convalescent serum, with RSV and influenza remaining the same on average. Patients with otherwise unknown aetiology show a small decrease.



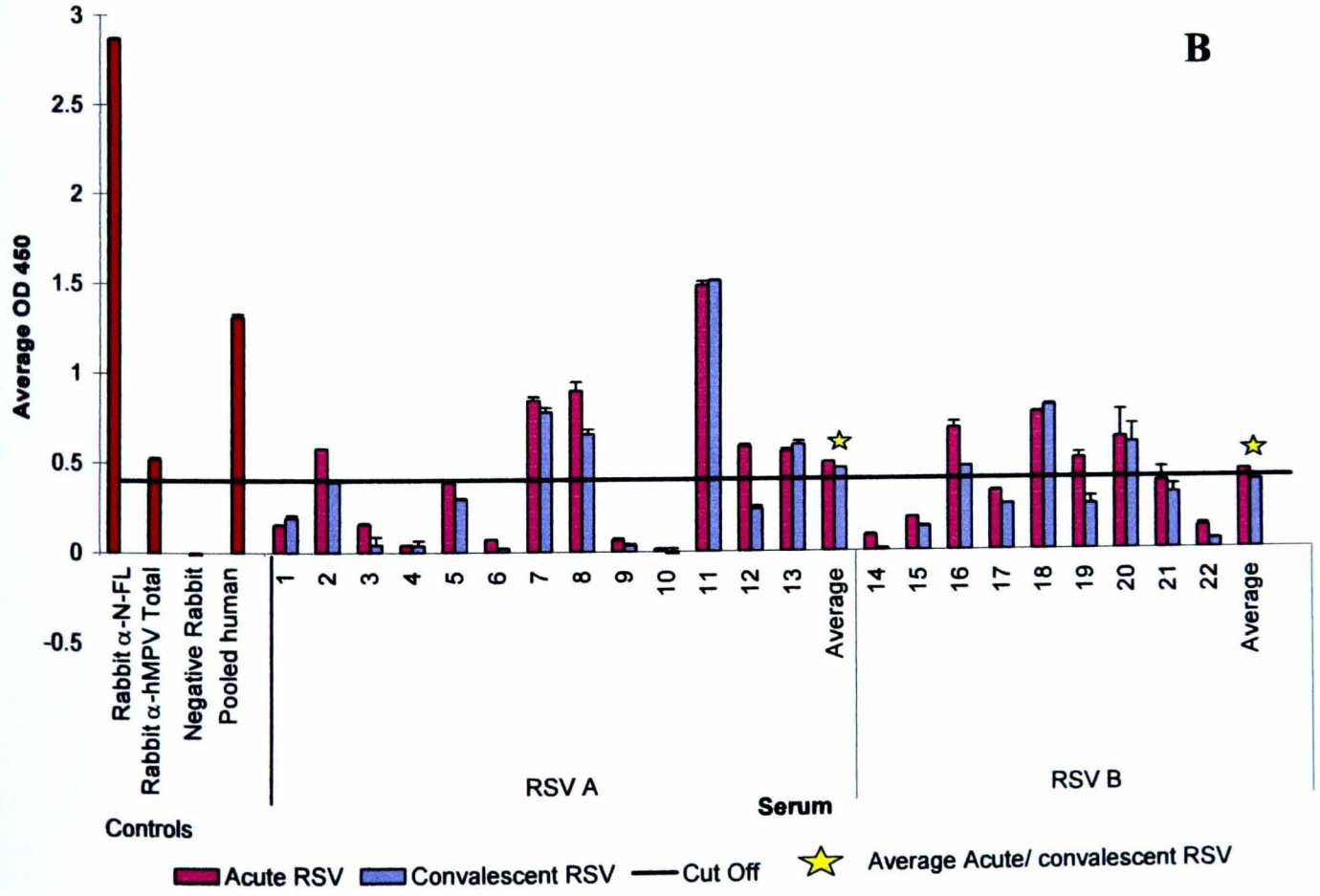
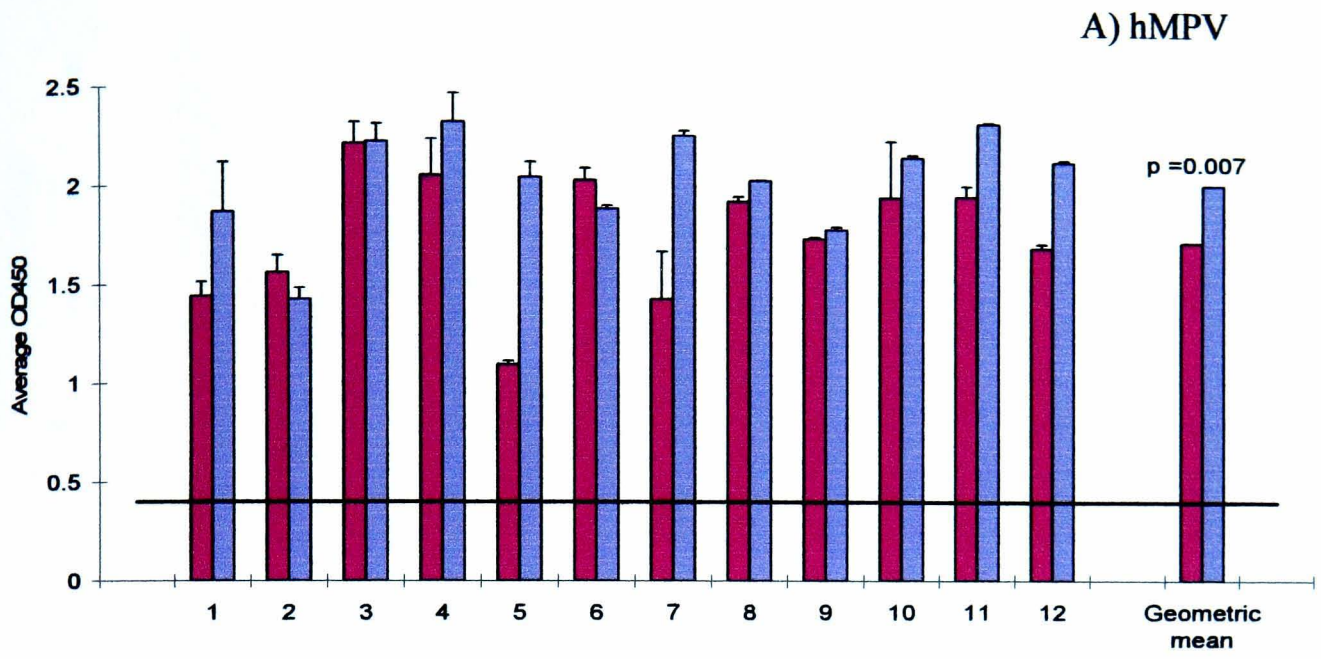


Figure 6.9: (A) The hMPV N ELISA reactivity of rabbit sera raised against a number of related respiratory viruses is shown as the corrected mean OD value in light blue, with the appropriate controls in red. (B) The hMPV N ELISA reactivity of 22 pairs of acute and convalescent primary RSV A or B infection sera from a paediatric population with the appropriate controls in Red. A yellow star highlights the average acute and

convalescent results for RSV A/ B infections. A horizontal black line indicates the 0.4 cut off. The OD value for the negative cell lysate was subtracted from the positive for each replicate (each was tested in triplicate) to give the corrected OD value. The average corrected OD value was then determined. Error bars show plus 1 Standard Deviation.



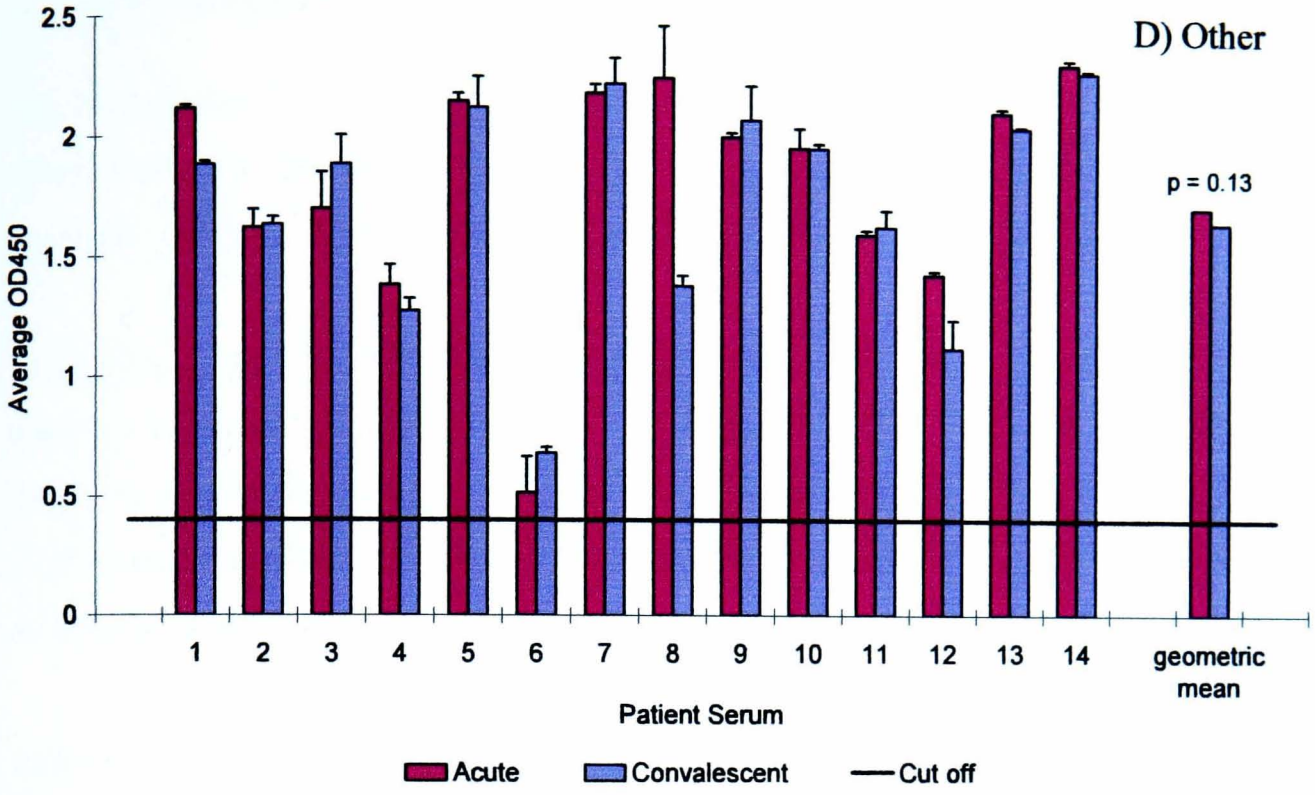


Figure 6.10: Analysis of sero-cross reactivity of acute and convalescent adult sera following (A) hMPV, (B) RSV, (C) Influenza, (D) Other viral infections, diagnosed by RT-PCR on corresponding combined nose and throat swabs taken during the acute phase of illness. Results show the corrected mean OD450 values for the acute (Blue) and convalescent (Red) serum at a 1:100 dilution performed in triplicate. Error bars indicate +1 Standard deviation. The black horizontal line indicates the cut off for the assay. P values calculated using a paired 2 sample student t-test. $P \leq 0.05$ is considered significant.

These findings support those previously published showing that sero-cross reactivity does not exists between hMPV and other common respiratory viruses including hRSV. There was no significant difference between the acute and convalescent sera from patients infected with RSV, influenza or other viruses, but there was significant difference in the OD values obtained between acute and convalescent sera collected from patients with hMPV infections. The hMPV N ELISA is therefore specific for the detection of Human and Rabbit α-hMPV N-FL IgG.

6.5 Seroprevalence in Age Stratified Sera from the UK

The assessment of the seroprevalence of hMPV in the UK population provides important information as to the age of primary infection, the age groups in which hMPV is most abundant, and who should be targeted for any potential hMPV vaccine regimes.

To do this a panel of age-stratified sera ranging from cord (taken at the time of delivery) to adult (>15 years) were tested by ELISA at a 1:100 dilution. The sera from the under 15 year olds were submitted to the HPA Colindale for investigation into the cause of unknown viral or rash like illness. The adult sera used were submitted to the HPA for investigation of influenza like illness.

Figure 6.11 shows the percentage of positive samples in each age group. The results show that at the time of birth (represented by cord sera) 100% of the samples tested were seropositive for hMPV. By the age of 6 months the number of positive samples decreases dropping to approximately 20% between the ages of 7 – 15 months. The percentage of positive samples then increased gradually from 16 months onwards. By the age of 6 years approximately 70 % of the samples were seropositive. This reaches 100% in the adult group.

These results indicate that hMPV infections occur throughout infancy, as the rate of seropositivity does not completely decline. As with hRSV, infections of hMPV early in infancy may be masked by the presence of maternal antibodies. A majority of primary infections, however, occur from the age of 16 months onwards with 70% of the population seeing an hMPV infection by the age of 6 years. This data therefore suggests that there is a notable difference in the acquisition of primary infection between individuals.

Furthermore this data indicates that by adulthood 100% of individuals have had an hMPV infection, yet data presented in chapter 3 shows that hMPV can cause sever influenza like illness in adults. This supports thinking that like RSV, hMPV has the ability to effectively evade host immune responses.

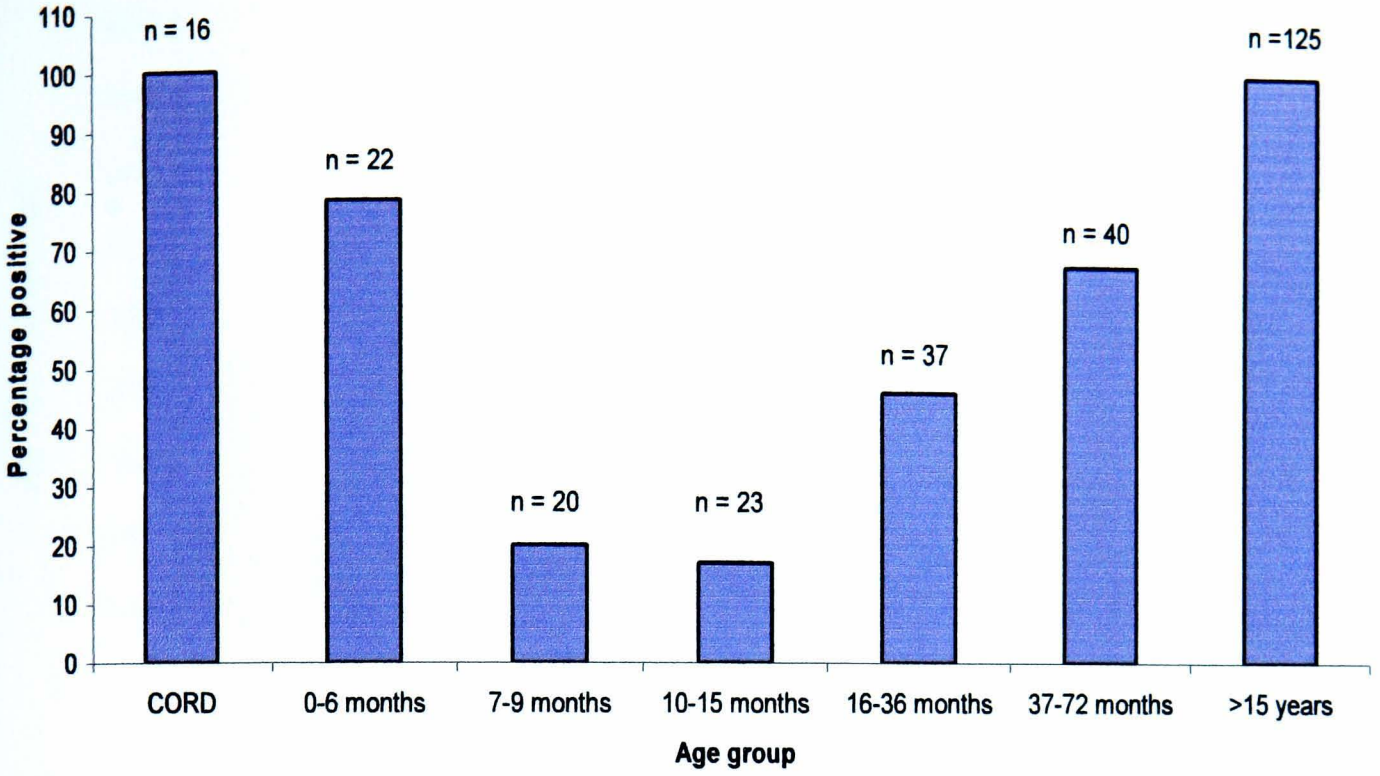
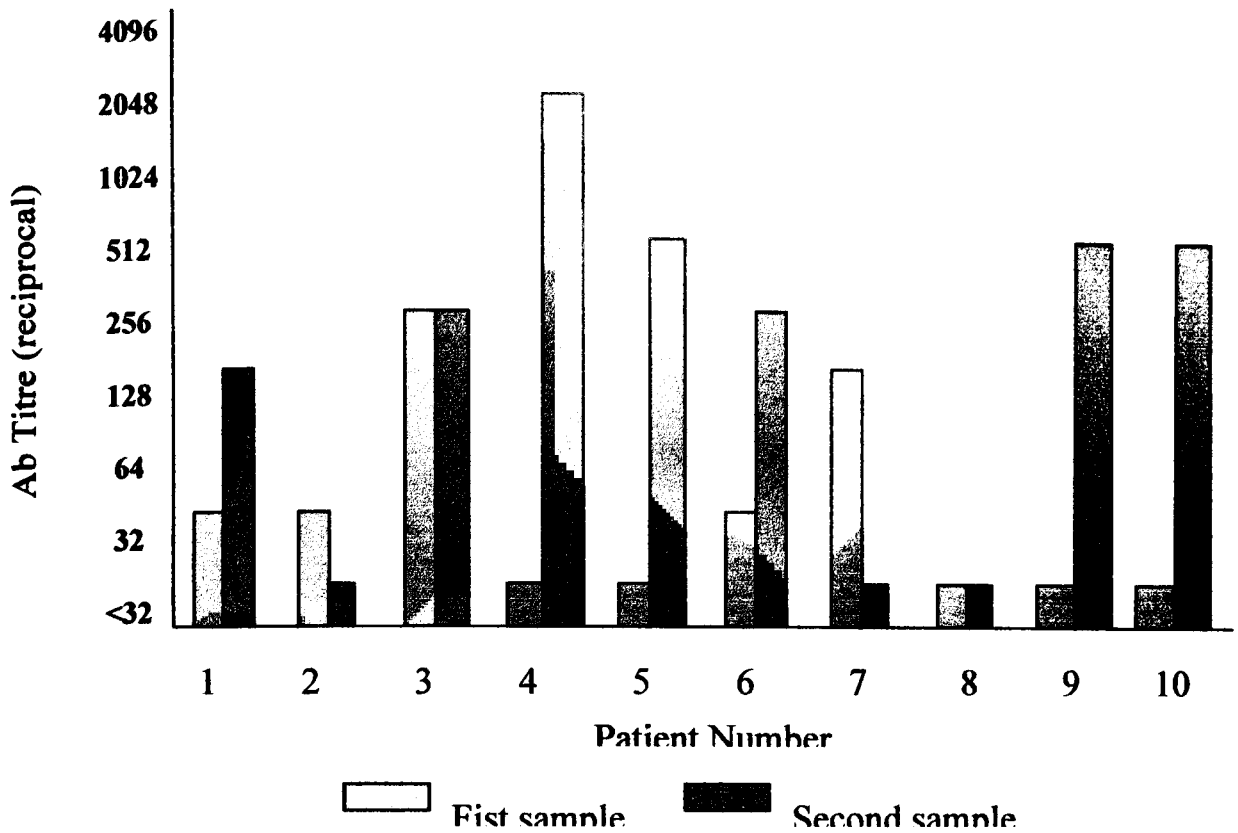


Figure 6.11: Sero prevalence of hMPV from a panel of age-stratified sera analysed by hMPV N ELISA. The percentage of positive samples is shown for each age group with n representing the number of samples tested in that group.

In addition to this 10 pairs of paediatric sere taken 12 months apart were analysed in a 2 fold dilution series in the hMPV N ELISA starting at 1:32. Of these 6 pairs sero-converted with a ≥ 4-fold increase in antibody titre. 2 pairs showed no change in Ab titre, while a further 2 showed a decrease.

Of those 6 that showed an increase in Ab, all were over the age of 16 months at the time the second sample was taken. This indicates that these individuals had had a primary infection with hMPV at some point over that 12-month period. Of the 4 that showed no change or a decrease in Ab titre all but one were under the age of 16 months at the time of the second sample, and as yet have not had a primary hMPV infection. The one individual which is the exception is patient number 7 who was 24 months old at the time the second sample was taken and infect shows an ≥ 8 fold decrease in Ab titre during that period.



Sample Number	Age at Time of First Sample (Months)	Age at Time of Second Sample (Months)
1	10	22
2	2	14
3	3	15
4	6	18
5	9	21
6	4	16
7	12	24
8	4	16
9	4	16
10	9	21

Figure 6.12: The hMPV N reciprocal Ab titre of 10 Pairs of paediatric serum taken 12 months apart analysed in the hMPV N ELISA in a two fold dilution series starting at 1:32. The table below shows the ages of the children in months at the time the first and second serum samples were taken.

To investigate further whether hMPV can cause severe influenza like illness in adults with pre-existing hMPV antibodies, acute and convalescent serum samples taken from around 100 community dwelling elderly patients with ILI were examined in the hMPV N ELISA. Of these, hMPV virus was identified in 12 of the corresponding combined nose and throat swabs by RT-PCR as described previously. The sera were tested in a 2 fold dilution series starting at 1:32 along with a subset of acute and convalescent sera following RSV infections from the same cohort as a control. Figure 6.13 shows the reciprocal antibody titre of the acute and convalescent sera for these 24 individuals.

As expected from the seroprevalence data above all patients were positive for hMPV N IgG at the time of infection. Of the 12 patients, which tested positive for an hMPV infection by RT-PCR, 5 show sero-conversion to hMPV following infection with a ≥ 4-fold increase in Ab titre. A further 4 patients have a 2 fold increase in Ab titre, while, one remains unchanged, and one shows a 2 fold decrease. Conversely, there is no change in hMPV Ab titre in individuals infected with hRSV, with the exception of RSV patients 9 and 12 in which there is a greater than 4 fold decrease in Ab titre.

The geometric mean titres of the acute and convalescent sera are 1290 and 3873 respectively. This indicates a 3-fold increase in antibody titre overall following an hMPV infection in this group of adults. P values indicate this is a significant increase. In comparison the geometric mean titres for acute and convalescent sera following RSV infection are 2050 and 1625.5 respectively which indicates a 1.2 fold decrease in Ab titre overall.

These findings support the above data by demonstrating the ability of hMPV to cause ILI in adults with pre-existing antibodies to hMPV and indicates that infection with hMPV results in an increase in hMPV N antibody titre in a majority of patients. A slight decrease in Ab titre following an RSV infection suggests that the increase is specific for hMPV and again indicates that there is no sero cross reactivity between hMPV and RSV.

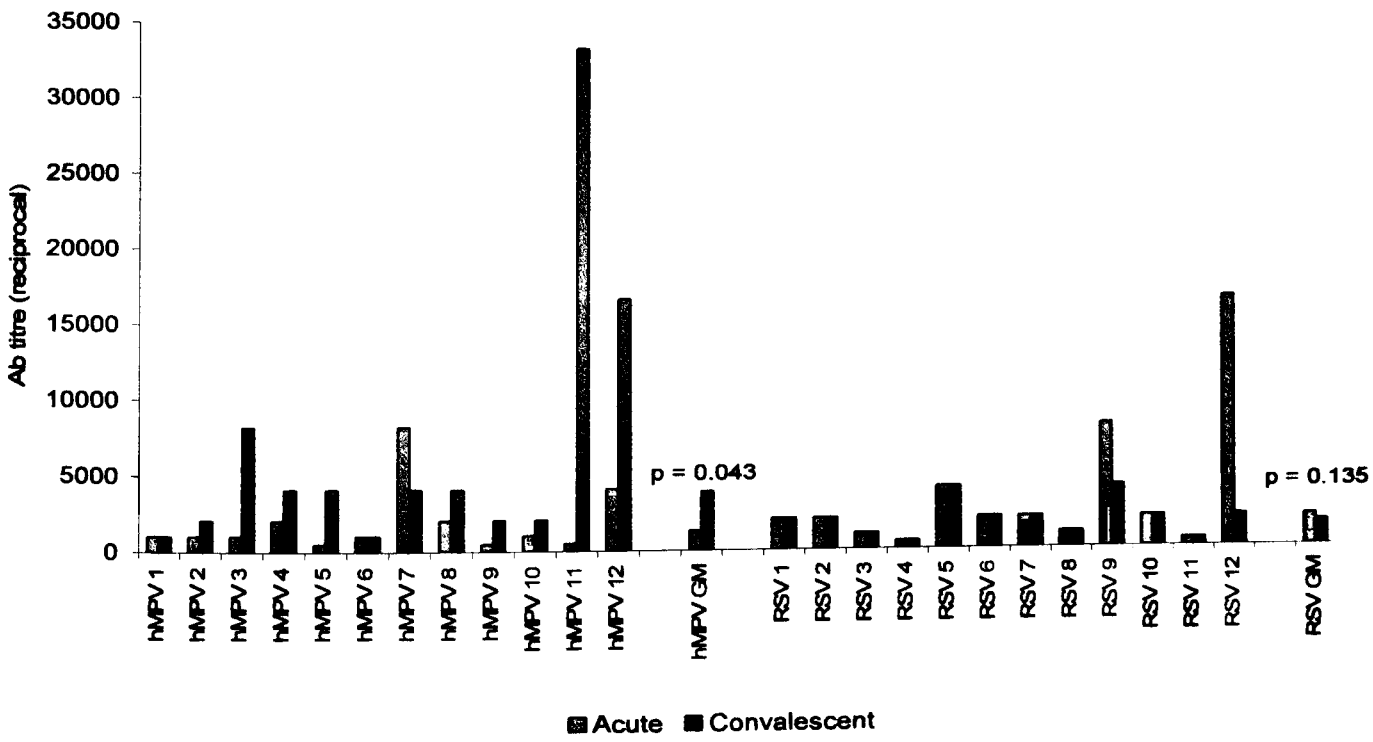


Figure 6.13: **The reciprocal antibody titre determined by hMPV N ELISA are shown for 12 pairs of acute and convalescent adult sera following hMPV or RSV infection and the corresponding geometric**

mean antibody titres . P values were calculated using a two-sample student t-test. $P \leq 0.05$ is considered significant.

6.5.1 Preliminary hMPV N Mapping

Further analysis of the human IgG Ab response against the hMPV N protein was performed against the different N fragments expressed in the recombinant baculovirus system, as described previously by immunofluorescence. This was done to enable preliminary analysis as to which regions of the hMPV N are most readily recognised by human Abs (Figure 6.14).

The results show that the hMPV N fragments A-E were expressed at roughly equal levels when analysed by the Mouse α–6-histitine MAb positive control serum (as in roughly the same number of fluorescing cells). The positive human serum was, as shown previously, reactive against the full-length hMPV N. It was also reactive against the hMPV N fragments D and E, and less so against fragment C and were virtually unreative against fragments A and B. The negative human serum was negative against the full-length hMPV N and each of the N fragments. A Similar pattern of reactivity was observed in western blot analysis of these fragments with Rabbit α-hMPV N at 1:250 shown also in Figure 6.14.

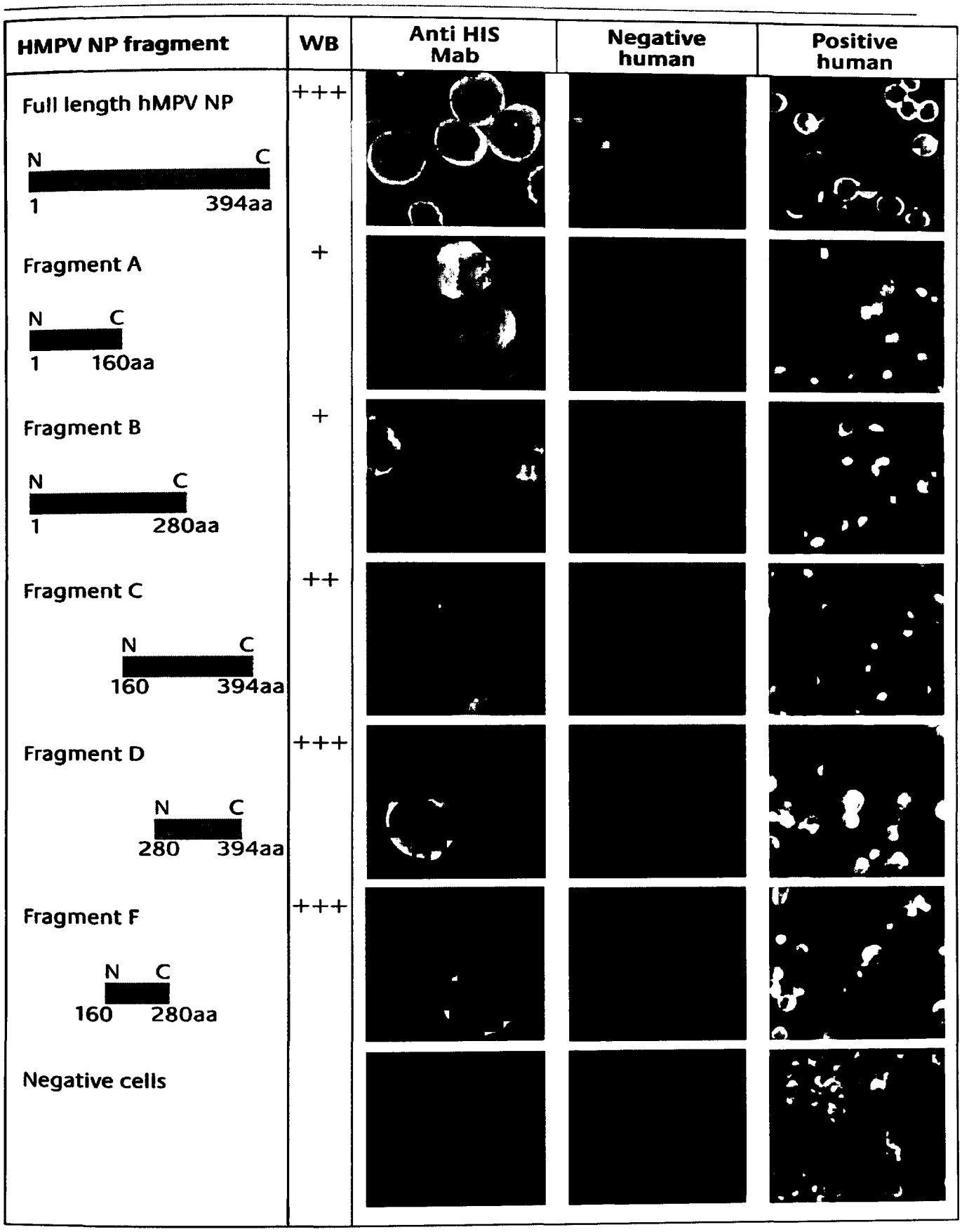


Figure 6.14: Preliminary mapping of human immune responses to hMPV N by Immunofluorescence. SF9 cells were grow on glass cover slips and infected with 3–40 MOI of the various recombinant baculoviruses, to ensure equal levels of protein expression for each of the hMPV N fragments. At 3 days post infection cells were acetone fixed and immuno-stained with human sera containing a high titre of hMPV N Ab, and a paediatric N negative serum diluted 1:25, both identified by the hMPV N ELISA. The bound Ab was detected with Mouse α-Human FITC conjugate at 1:40. As described previously each fragment contained an N terminal 6-histadine tag, and therefore a Mouse α–6-histitine MAb 1:50 was used as a positive control

6.6 Discussion

Reported here is the development and validation of an ELISA for the specific detection of human α-hMPV N antibodies, based on the recombinant baculovirus expressed hMPV N-FL protein. Also reported is the application of the ELISA to produce the first data on the seroprevalence of hMPV in the UK, and evidence of repeated hMPV infections occurring in adults despite the presence of pre-existing hMPV antibodies.

The results demonstrate that the recombinant baculovirus expressed hMPV N-FL protein can serve not only as an immunogen for the preparation of hMPV specific antiserum, as described in chapter 5, but also as an abundant source of antigen for the development of serological assays and the measurement of human antibodies to hMPV.

The hMPV N protein was chosen as the appropriate antigen due to the low level of amino acid variation observed between the N proteins of hMPV strains belonging to the A and B lineages. Published findings of hMPV N sequence analysis shows that there is between 98.3 -100% amino acid homology within lineages and 94.2 -95.9% between lineages (Bastien et al., 2003, van den Hoogen et al., 2002). A high degree of sero-cross reactivity between the N protein of viruses belonging to both lineages has also been demonstrated (Hamelin & Boivin, 2005).

To evaluate the specificity of the N ELISA, antiserum raised against a number of other common respiratory viruses, including hRSV was analysed. The results presented here indicate that there is no sero-cross reactivity between hMPV and other human respiratory viruses, including hRSV, supporting previously published data (Alvarez et al., 2004, van den Hoogen et al., 2001). Work presented in this thesis provides further support of these findings as acute and convalescent paediatric sera taken from children with primary RSV infections showed no increase in the OD value of the convalescent serum in the hMPV N ELISA. Unfortunately, due to the limited availability of the sera it was not possible to look for changes to antibody titre, which would have given a more accurate indication of any cross-reactivity that may have existed. Analysis of the hMPV N antibody titres of acute and convalescent RSV adult serum, however was investigated, and in fact showed an overall decrease in hMPV antibody titre in the hRSV infected patients.

Analysis of a panel of age-stratified sera from the UK shows that hMPV is a common childhood pathogen with approximately 70% of children being infected by the age of 6 years which is consistent with recent published findings (Ebihara et al., 2003, van den Hoogen et al., 2001, Wolf et al., 2003). At the time of birth sera collected from the cord is a good indication of the antibody status of the infant at that time. The data presented here shows that 100% of the population had antibodies to hMPV at the time of birth, and this is likely to reflect the presence of transplacentally acquired maternal antibodies. By the age of 7 months only 20% of the samples tested had antibodies to hMPV, which represents a decline in the maternal antibody levels. The percentage of individuals positive for hMPV antibodies remained at just below 20% until the age of 16 months when the rate of positivty began to gradually increase. Similar findings were observed by Wolf *et al* (2003) who analysed sera by IF of hMPV infected cells (Wolf et al., 2003).

As positivity does not completely decline these results indicate that primary hMPV infections occur throughout infancy and like hRSV, infections occurring in very young children may be masked by the presence of maternal antibodies (Glezen et al., 1981, Ogilvie et al., 1981).

In a vast majority of cases primary hMPV infections occur in children who are 16 months or older. By the age of 6 years approximately 70% of the cohort had had an hMPV infection. This data supports previously published findings carried out by other groups using IF, and other ELISA techniques (Boivin et al., 2002, Ebihara et al., 2004a, Ebihara et al., 2003, Falsey et al., 2003, Hamelin & Boivin, 2005, Leung et al., 2005, van den Hoogen et al., 2001, Wolf et al., 2003). This data indicates that there is a wide range in the age at which children have primary hMPV infections and acquire immunity, however, when compared with primary hRSV infections, primary hMPV infections appear to occur in slightly older children, with a majority of individuals having experienced hRSV infection by the age of 2 years (Ebihara et al., 2004b, Glezen et al., 1986).

The data also suggests that children aged between 7-16 months could be targeted for potential hMPV vaccines regimes, without interference from maternal antibodies (Crowe, 2001). Whilst vaccination at this stage will not benefit the estimated 20% of the population who experience hMPV infections early during infancy, it may benefit the other

80%. However, debate exists, as with hRSV, as to the efficacy of a vaccine for these viruses due to the level of variation that exists within the G glycoprotein (chapter 4), and the ability of hMPV to cause repeat infections.

Analysis of sera from adults indicates virtually all adults have detectable levels of hMPV N specific antibodies, as reported elsewhere (Falsey et al., 2003, Hamelin & Boivin, 2005). However, the data presented in Chapter 3 demonstrates that a substantial number of adults experience severe hMPV infections. Furthermore, analysis of paired sera from adults with severe hMPV infections in this chapter demonstrates the ability of hMPV to cause repeated infections despite the presence of existing hMPV antibodies and that hMPV infections result in an increase in antibody titre in a majority of adults.

In a recent study conducted by Hamelin *et al* (2005) recombinant nucleocapsid protein expressed in *E.coli* was used to develop an hMPV specific ELISA. Their data demonstrated that children had higher antibody titres than adults, and suggested that adults may loose their antibodies to hMPV, or that they may fall below detectable levels for their assay (Hamelin & Boivin, 2005). The antibody titres of children and adults were not directly compared in this study and it would be an interesting angle to pursue in future work addressing the role of immunity in hMPV reinfection. These data highlight the need for more extensive research to be carried out on hMPV immune evasion mechanisms, the analysis of strains circulating in the community, the induction and longevity of neutralising antibodies to hMPV and its different proteins.

The data presented in chapter 4 shows that the hMPV G a major surface antigen, displays a very high degree heterogeneity, and it is postulated that this protein plays an important role in immune evasion, preliminary work looking at the antigenic analysis of hMPV G to try and address some of the questions raised throughout this work is presented in Chapter 7

Analysis of post infection sera for its ability to neutralise virus will also provide a lot of information regarding the induction of immunity. Neutralising antibody levels cannot be measured by ELISA and instead requires functional assays such neutralisation or plaque reduction assays. Establishing such assays however may be problematic given the

difficulties associated with growing hMPV in the laboratory, and through evaluation of the assay is required. Recently, Biacchesi *et al* (2005) have reported the development of a microtitre neutralisation assay for hMPV based on the rescue of hMPV from cDNA (Biacchesi et al., 2005). This will provide a useful tool of analysing overall neutralising antibody responses to hMPV. The contribution of the individual surface glycoprotein's of hMPV to neutralisation have also been investigated through the use of chimeric viruses. The published data indicates that a majority of neutralising antibody is targeted towards the F protein (Skiadopoulos et al., 2006). However as yet only sera from experimentally infected animals have been evaluated in these assays and extensive evaluation of human sera reactivity should be conducted.

Preliminary data of the human Ab reactivity to different fragments of the N was also presented. A majority of human Ab reactivity by IF and WB appeared to be targeted against fragments D and E which are located at the carboxyl terminal of the N protein. This however is very provisional data, and further investigation by Western Blot analysis and ELISA and FACS, is required, and will provide not only immunologically relative information but can also infer information on the structure of the N. Previous work by Ryan *et al* used MAb mapping techniques to define the regions of paramyxiovirus nucleocapsids important for immunogenicity and nucleocapsid assembly. They found that for PIV3 and Sendai virus the carboxyl terminal of the N contained a majority of the antigenic sites [(REF)], which is consistent with the preliminary findings presented here. The identification of immunodominant epitopes may enable further refinement of immune-assays targeted against the N through the use of peptides.

In summary, reported here is a simple and specific ELISA for the detection of human antibodies to the hMPV protein, and its application investigate the seroprevalence of hMPV in the UK. Although not a useful tool for diagnosis of hMPV during the acute phase of illness, this ELISA may be useful as a confirmatory test during outbreak situations through the analysis of acute and convalescent serum, or for further investigation of seroprevalence.

Chapter 7

Recombinant baculovirus expression of hMPV G

7.1 Introduction

The sequence and phylogenetic analysis of the hMPV attachment glycoprotein of circulating strains presented in chapter 4 raises some important questions regarding the structure, function and immunogenicity of the protein.

The high degree of amino acid variation suggests that G plays an important role in the evasion of host immune responses. The analysis of human Ab reactivity in terms of cross-reactivity, epitope mapping, neutralizing ability, and the effect of such Ab on the sequence diversity of G should all be addressed.

In order to do so however the basic reagents such as an appropriate hMPV G antigen and hMPV G specific antiserum are required. This chapter details the development of such reagents through the expression of hMPV G in the recombinant baculovirus system.

There have been numerous examples of the attachment glycoproteins of closely related viruses such as RSV and APV, as well as the surface glycoproteins of other viruses, such as the influenza haemagglutinin being successfully expressed in the recombinant baculovirus system and used for the development of immunoassays and protein characterisation and vaccination (Buraphacheep et al., 1997, Crawford et al., 1999, Sugiura et al., 2001). The major disadvantage of using the baculovirus system for a protein that is expected to be heavily glycosylated (Chapter 4) is that the glycosylation provided in the insect cells is different in its extent and nature compared to that in mammalian cells (Jarvis, 1997). Work published by Sullender and Britt showed that the RSV attachment glycoprotein expressed in the baculovirus system had reduced levels of glycosylation when compared to RSV G expressed in mammalian cells. Despite this, the localisation and antigenicity of the baculovirus expressed protein was maintained reacting against both monoclonal antibodies raised against native G, and human polyclonal serum. Furthermore, they demonstrated that the inoculation of cotton rats with the recombinant G protein conferred protection against wild type RSV challenge (Sullender & Britt, 1996). These data therefore support the use of the baculovirus system for the immune-analysis of the hMPV attachment glycoprotein.

7.2 Aims

- Expression of the hMPV G protein in the recombinant baculovirus system as an alternative to G purified from hMPV infected cells.
- Generate high titre hMPV G specific polyclonal antiserum.
- To evaluate the potential use of the G antigen and antiserum as tools for the assessment of serological response.
- Investigation in to human antibody reactivity against the hMPV G protein, as provisional data for the investigation of the antigenic properties of the G protein. What is the prevalence of hMPV G antibody in the UK population? Are antibodies to G cross-reactive? What evidence is there supporting positive selection of mutations occurring in this protein? Is there evidence that glycosylation interferes with antibody recognition of this protein?

7.3 Recombinant Baculovirus System

Two recombinant baculoviruses expressing hMPV G were constructed, the first expressing the full length attachment glycoprotein (G-FL) containing a 6 histidine tag at the amino terminal, and the second, a secreted form of hMPV fused to the Fc fragment of human IgG containing a 6 histidine tag at the carboxyl terminal of the fusion protein (G(s)-Fc) (Figure 7.2).

For expression of G-FL the commercially available plasmid; pTriEx-2 Neo; used previously for the hMPV N protein expression work, was used as the baculovirus transfer vector and mammalian expression system.

For expression of G(s)-Fc a second transfer vector was used. This vector is based on the pTriEx vector system with the addition of the human IgG Fc fusion protein. The use of the Fc fragment as a fusion protein has been used previously and shown to aid in the expression of secreted proteins by improving cellular processing and increasing the solubility of the protein (Schutze et al., 2005, Sugiura, 2003)

G-FL and G(s) were amplified by PCR from the same isolate used in the hMPV N work presented in chapters 5 and 6, using primers containing the appropriate restriction sites (Table 7.2 and Figure 7.1). PCR products were cloned in to the TOPO cloning vector, and subsequently sub-cloned into the appropriate transfer vector (Figure 7.2).

Based on the expression of other membrane proteins in the baculovirus system, it was expected that G-FL would be expressed on the surface of the cell, and G(s)-Fc is secreted in to the cell culture medium.

Table 7.1 Primers used for the amplification of the hMPV G gene and G(s) fragment for cloning into the pTriEX vector.

Primer	Position*	Sense	Sequence 5'-3'
hMPVG BamH1F	6247-6267	+	GGATCCTATGGAGGTGAAAGTGGAGAA
hMPVG BamH1StopR	6956-6937	-	GGATCCTGTTAACTAGTTTGGTTGTATG
hMPVG (S) BacF	6406-6421	+	GGCCATTATGGCCATACAATGCAAGAAA AC
hMPVG (S) BacR	6956-6937	-	GGCCGAGGCGGCCACTAGTTTGGTTGT ATGTTGTTGA

Footnotes: (*) primer positions according to reference strain NL00-1.

Table 7.2: Primers, restriction enzymes, and vectors used for the construction of pTriEx mammalian expression and baculovirus transfer vectors.

Gene / Gene Fragment	G -FL	G (S)
Forward Primer	hMPVGBamH1F	hMPVG(S)BACF
Reverse Primer	hMPVGBamH1STOPR	HMPVG(S)BACR
Expected Size (bp)	726	562
Restriction Sites	BamH1	Sfi1
Expression Vector	pTriEx-2 Neo	PTriEx- IgG Fc
Recombinant Baculovirus	Baculovirus - hMPV G-FL	Baculovirus - hMPV G (s)-Fc
Expected Recombinant Protein Size (KDa)	32.8*	50.6*

Footnotes: (*) The molecular weight of the proteins was estimated for the non-glycosylated species from amino acid sequences using EditSeq, DNAStar Lasergene software. For primer sequences see Table 7.1.

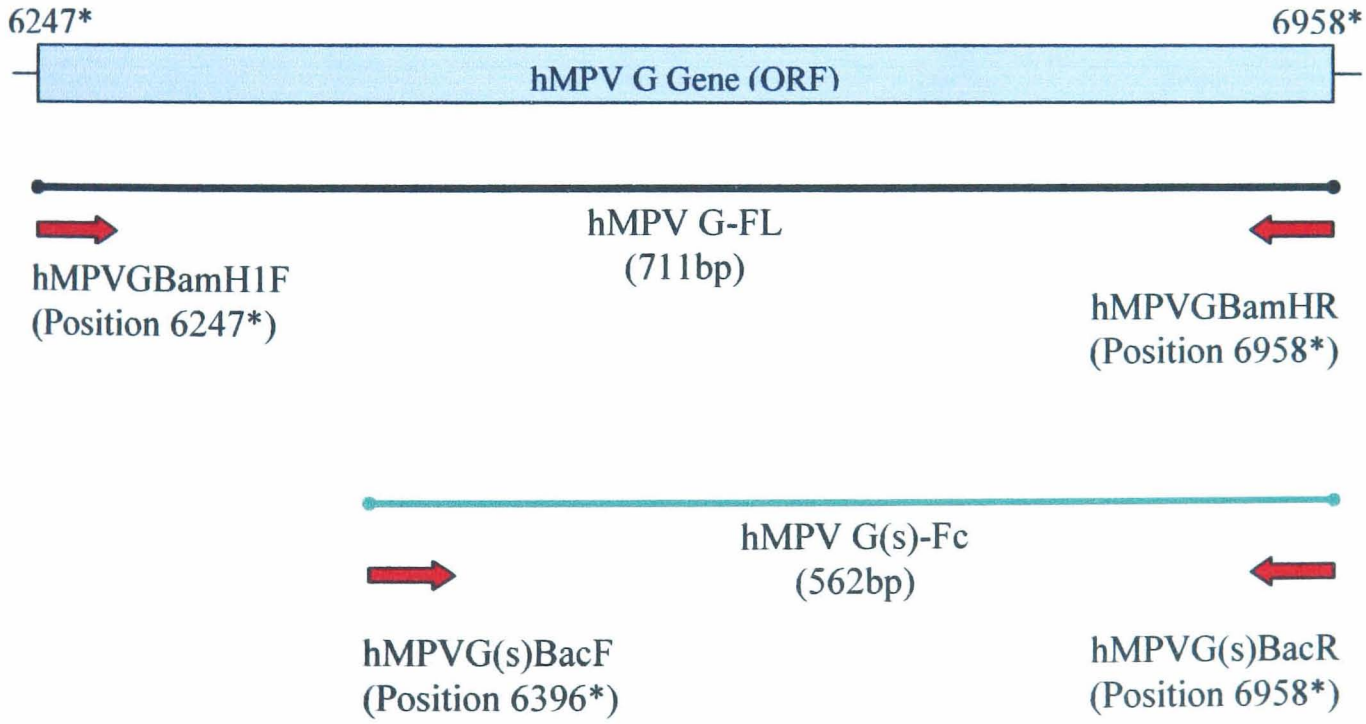


Figure 7.1: Schematic representation of the amplified hMPV G regions and primer positions. The blue box represents the hMPV G gene ORF. The black line represents G-FL and the green line G(s). The size of each G region amplified in indicated in base pairs (bp) below the coloured line. Primers are represented by red arrows with the name of the primer below and the nucleotide starting position for the primer. (*) starting primer positions according to reference strain NL00-1. For primer sequences see Table 7.1.

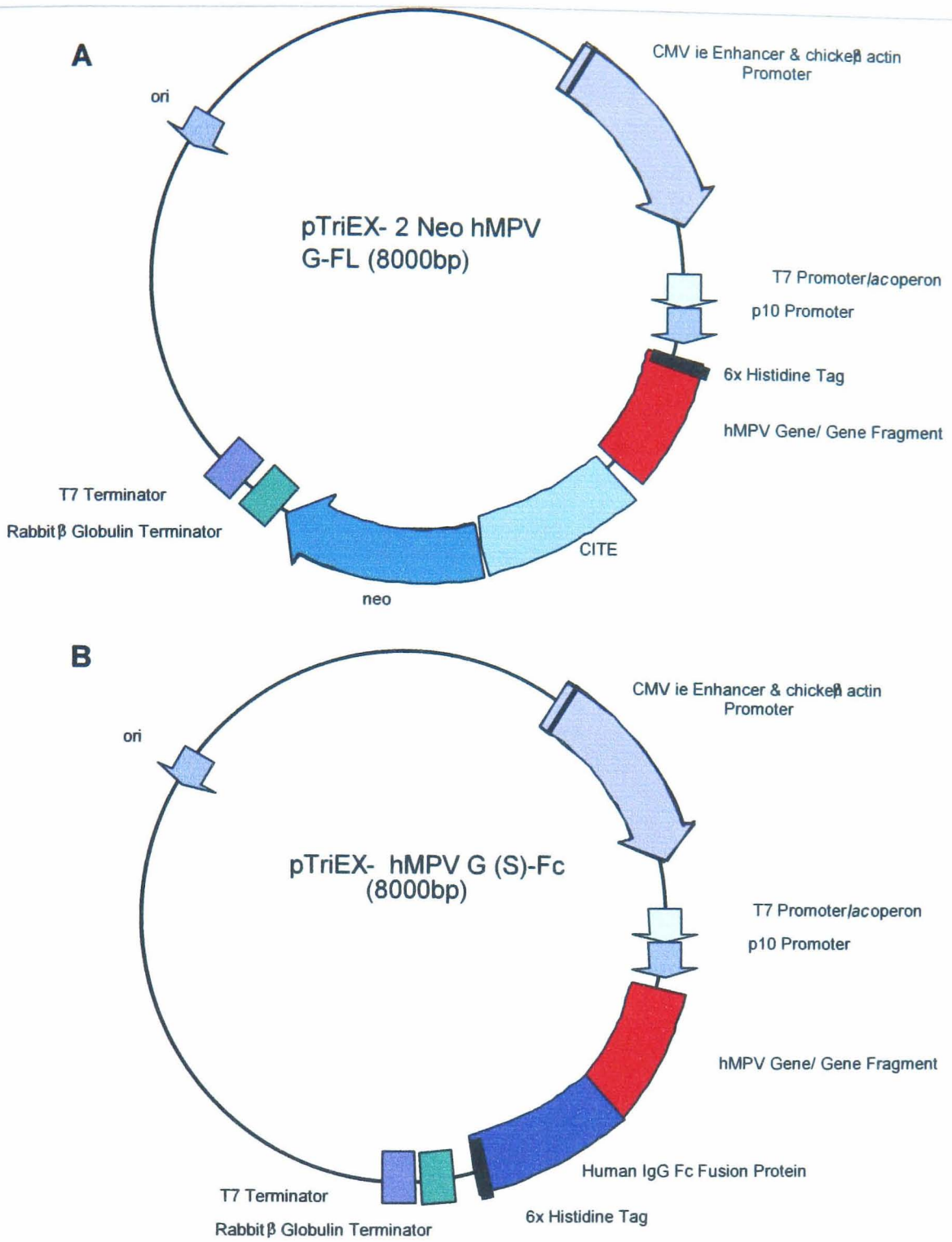


Figure 7.2: Schematic representation of the hMPV pTriEx G-FL and G(s)-Fc constructs used as transfer vectors for generating the recombinant baculoviruses. (A) hMPV G-FL, and (B) hMPV G(s)-Fc. Each construct contains a CMV ie enhancer and chicken β-actin promoter for expression in mammalian systems (purple), a T7 promoter for bacterial expression (pale green), and the p10 promoter for recombinant baculovirus expression (pale blue). 6x histidine tags (Black) are available in both vectors these are located just upstream of the multiple cloning site for G-FL and immediately down stream of the Fc fusion protein (blue) in the G(s) construct. The position of the inserted hMPV gene is shown in red. Both vectors contain the Rabbit β-globulin (green) and T7 terminator (dark blue). The G-FL construct also contains the neomycin resistance gene for the selection of stable mammalian cell lines which operates under the control of the EMCV-derived Cap-Independent Translation Enhancer (CITE).

Table 7.3: Details of the hMPV virus strains used as antigen in this chapter.

Antigen	Source	Lineage
G-FL and G(s)-Fc	4-year-old male diagnosed with bronchiolitis and upper respiratory tract infection in 2002, collected as part of the investigation in to the cause of illness in children hospitalised with ARTI in the UK isolated in LLC-MK2 cells.	A1 (UK324-02) *
hMPV infected cells	Northern Ireland HPA (2004) investigation in to the cause of respiratory illness first isolated in RMK cells. Further propagated at Colindale HPA in LLC-MK2 cells by Dr Alison Bermingham.	A1 (NI-04) B1 (NI2-04)

Footnotes: (*) see Chapter 4 for positioning in phylogenetic tree and alignment described as hMPV G clone.

Table 7.4: Details of the hMPV specific rabbit serum used in this chapter.

Antiserum	Source of antiserum	Source of Antigen	Lineage
Rabbit α-hMPV Total	Colindale HPA raised against an hMPV infected RMK cells lysate isolated by Dr Joanne Stockton.	From child < 5 years collected as part of the investigation in to the cause of illness in children hospitalised with ARTI in the UK.	A (UK241-01)
Rabbit α-hMPV G(s)-Fc	This study, raised against recombinant baculovirus expressed hMPV G(s)-Fc High 5 cell culture supernatant	Recombinant baculovirus expressed hMPV G(s)-Fc generated in this study. See table 7.3 for further detail.	A1 (UK324-02) *

Footnotes: (*) see Chapter 4 for positioning in phylogenetic tree and alignment described as hMPV G clone.

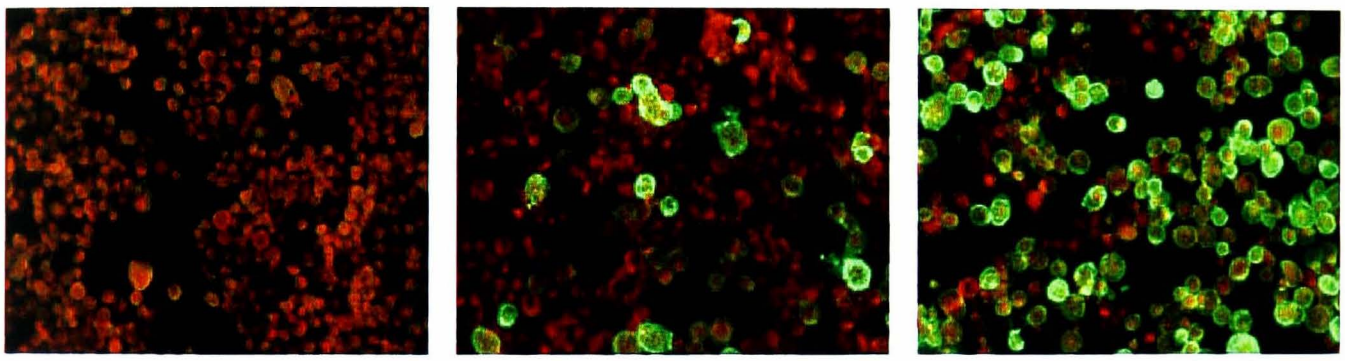
Table 7.5: Details of the human serum used in this chapter.

Human serum	Source
Pooled human sera	Colindale HPA (2001) pooled adult sera negative for reactivity against influenza antigens submitted to the HPA for serological investigation of respiratory illness.
Positive human serum	Colindale HPA (2001-2004) adult sera submitted to the HPA for serological investigation of influenza.
Negative human serum	Colindale HPA. Neonatal paediatric sera collected as part of a longitudinal cohort study of RSV infection in childhood (Althani, 2004).

7.4 Recombinant Baculovirus Expression of hMPV G-FL

The recombinant baculovirus expressing the full-length hMPV attachment glycoprotein (G-FL) was generated as described previously (Chapter 5).

To monitor the expression of hMPV G-FL immunofluorescence (IF) analysis was performed on SF9 cells grown on glass cover slips and infected with approximately 10 - 20 MOI of the recombinant baculovirus. Cells were fixed with acetone and the expressed protein was detected using a mouse α-6 histidine MAb (1:50) followed by rabbit α- mouse IgG FITC conjugate (1:40) and visualised by fluorescence microscopy (Figure 7.3). The results show expression of the hMPV G-FL protein in the infected cells. The level of expression was considerably greater in cells infected with 20 MOI of virus compared to those infected with an MOI of 10.



A: Uninfected **B:** 10 MOI **C:** 20 MOI

Figure 7.3: Immunofluorescence analysis of recombinant baculovirus hMPV G–FL. (A) uninfected, (B) infected at 10 MOI, or (C) infected at 30 MOI of virus. Detected with mouse α-6-histidine MAb (1:50) followed by rabbit α-Mouse IgG FITC conjugate (1:40.)

Initial analysis of the clarified cell lysate by western blot using the mouse α-6-histidine MAb failed to detect any protein. Therefore analysis of the different cell lysate fractions was carried out. SF9 cells were inoculated with recombinant baculovirus hMPV G-FL at an MOI of 5, 10, 20 or 40 in a shaker culture. Cells were harvested 3 days pi and lysed using insect pop culture reagent (Novagen). The cleared cell lysate, and the cell debris was analysed by western blot. In accordance with the IF results G-FL was detected in cells infected with 10 or 20 MOI of virus (Figure 7.4) protein expression was not detected at an MOI of 5 or 40 (data not shown).

Lanes 1 and 2 contain the cell lysate supernatant, and were negative for G-FL. Lanes 3 and 4 contain the cell debris, and are positive for G-FL, indicating the lysis or solubilisation

was insufficient to release the protein from the cellular membrane material. This result suggests that the expressed protein is targeted correctly within the cell to either the plasma membrane or within the endoplasmic reticulum or Golgi apparatus for which different detergent may be required for lysis. Two bands are visible in both lanes 3 and 4. The low molecular weight band (LMW) represents the correct size for the un-glycosylated species and the high molecular weight band (HMW) most likely representing various glycosylated species; a pattern typically observed for hMPV and RSV attachment glycoproteins, although the presence of glycosylation was not investigated in this work.

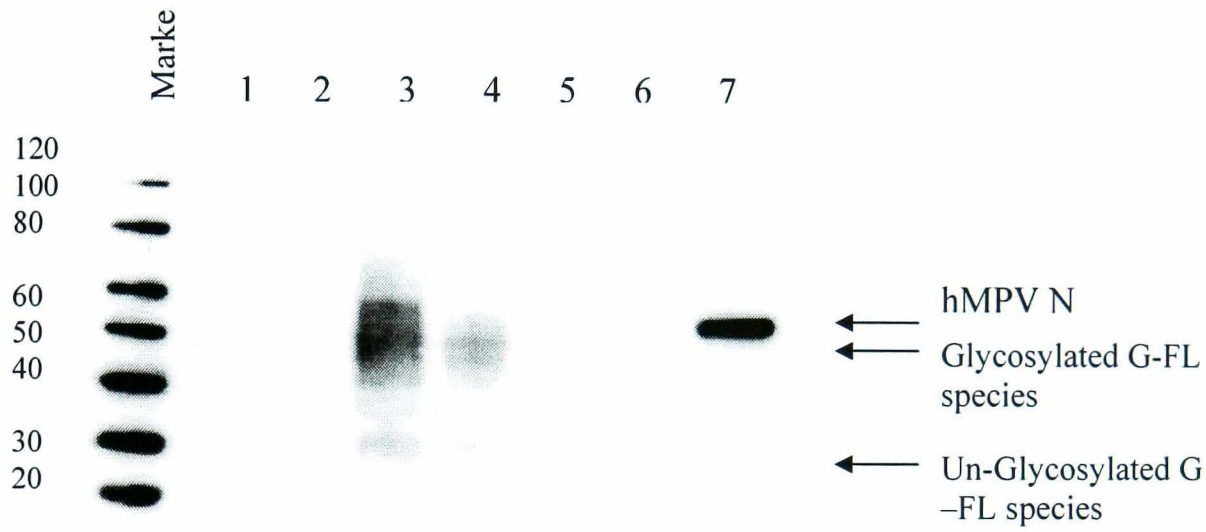


Figure 7.4: Western Blot analysis of different cell lysate fractions of SF9 cells infected with Baculovirus hMPV G-FL at different MOI. Mouse α-6 histidine MAb 1:1000 and Rabbit α-Mouse HRP conjugate 1:2500. (1) hMPV G-FL cell lysate supernatant MOI 20, (2) hMPV G-FL cell lysate supernatant MIO 10, (3) hMPV G-FL cell debris MOI 20, (4) hMPV G-FL cell debris MOI 10, (5) Uninfected cell lysate supernatant, (6) Uninfected cell debris, (7) Purified hMPV N Positive Control.

To improve the solubilisation of membrane proteins and determine the optimal day for harvesting the expressed G-FL protein, a time course assay was performed. SF9 cells were infected with 20 MOI of recombinant virus and samples collected every 24 hours. Cells were collected by centrifugation; freeze thawed and lysed using a number of different non-ionic detergents, including 1% NP-40, 0.1% Triton X-100, and 2% Tween-20. The lysed cell preparations were clarified by centrifugation and analysed by Western blot analysis (Figure 7.5).

It was important that non-ionic detergents were used in order to prevent denaturation of the protein which would subsequently be used for antibody production and immunoanalysis.

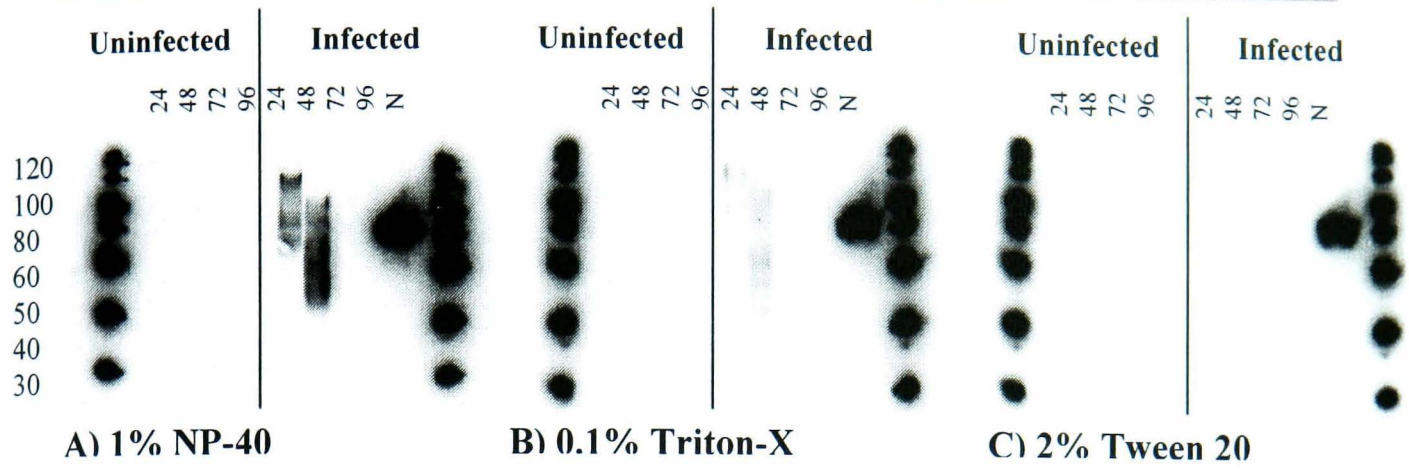


Figure 7.5: Western Blot analysis of cell lysate supernatant taken from recombinant baculovirus hMPV G-FL infected SF9 cells at an MOI of 20 over a time course of 24, 48, 72 and 96 hours post infection. (N) Recombinant baculovirus expressed hMPV N-FL used as Western blot positive control antigen. (A) Cells lysed with 1% NP-40 and (B) cells lysed with 0.1%^ Triton X 100. (C) Cells lysed with 2% Tween 20. Detected using a mouse α-6-histidine MAb followed by rabbit α-mouse IgG HRP conjugate.

Results show that lysis using 2% Tween-20 released only very small quantities of G-FL from the membranous fractions, while cells lysed with 1% NP40 or 0.1% Triton X-100 were successful at 24 and 48 hours pi. NP-40 yielded the largest amount of protein (Figure 7.5 A) The species detected resulted in a smeared appearance that differed in size between the 2 time points and likely represents different levels of posttranslational modification (e.g. glycosylation). Compared with Figure 7.4 in which the membranous fraction was analysed there was greater heterogeneity in the size of the proteins analysed in Figure 7.5.In particular HMW species were more viable indicating species with a higher degree of O-linked glycosylation were present in this antigen preparation.

In summary the hMPV full-length attachment glycoprotein was expressed at detectable levels in SF9 cells when a high MOI of recombinant hMPV G-FL baculovirus was used. The protein however was only separated efficiently from cellular material with NP40 resulting in a protein preparation with a high degree of molecular weight heterogeneity. Due to the high MOI required in order to achieve detectable levels of baculovirus expressed G-FL, virus purification using a sucrose gradient was attempted but was unsuccessful (data not shown). The quantities of the expressed protein required for protein purification, and the generation of G-FL specific polyclonal antiserum based on purified G-FL was therefore considered unfeasible.

7.5 Recombinant baculovirus expression of hMPV G(s)

As discussed in chapter 4, the G protein of RSV is produced not only in the full length transmembrane form, but also in a secreted form in which the cytoplasmic and transmembrane regions of the protein are deleted. This arises due to initiation of translation at a second methionine codon in the same ORF, situated immediately after the transmembrane region. A second in-frame methionine codon is also present at a similar location in the G gene of hMPV. This suggests that a secreted version of hMPV G may also be produced during hMPV viral infection, although this is yet to be investigated. However, expression of a secreted version of G may overcome the problems associated with producing the large enough quantities of G-FL for antibody production.

The secreted version of the attachment glycoprotein (G(s)) was generated by amplifying the region from the second methionine codon to the stop codon at the end of the ORF, therefore excluding the cytoplasmic and transmembrane regions of the protein (Figure 7.1). This protein was fused at the carboxyl terminal of the protein to the Fc fragment of human IgG to increase solubility and help maintain the correct folding and processing of the truncated G protein.

Although work presented in chapter 4 suggests that the carboxyl terminal of G is unlikely to play an important role in the structure or function of the molecule, this has not been investigated further and mapping of the major epitopes of this protein is still yet to be reported. DNASTAR software was therefore used to assess how the presence of the Fc fusion protein might affect the secondary structure of G (Figure 7.6).

Results show that the IgG-Fc fusion protein appears to have little effect on the predicted secondary structure, hydrophilicity or surface probability profiles of the G region when compared to that of the G-FL over a range of different window sizes (5-20 amino acids). A recombinant baculovirus expressing a secreted version of the hMPV attachment glycoprotein with the IgG-Fc fusion protein was therefore generated.

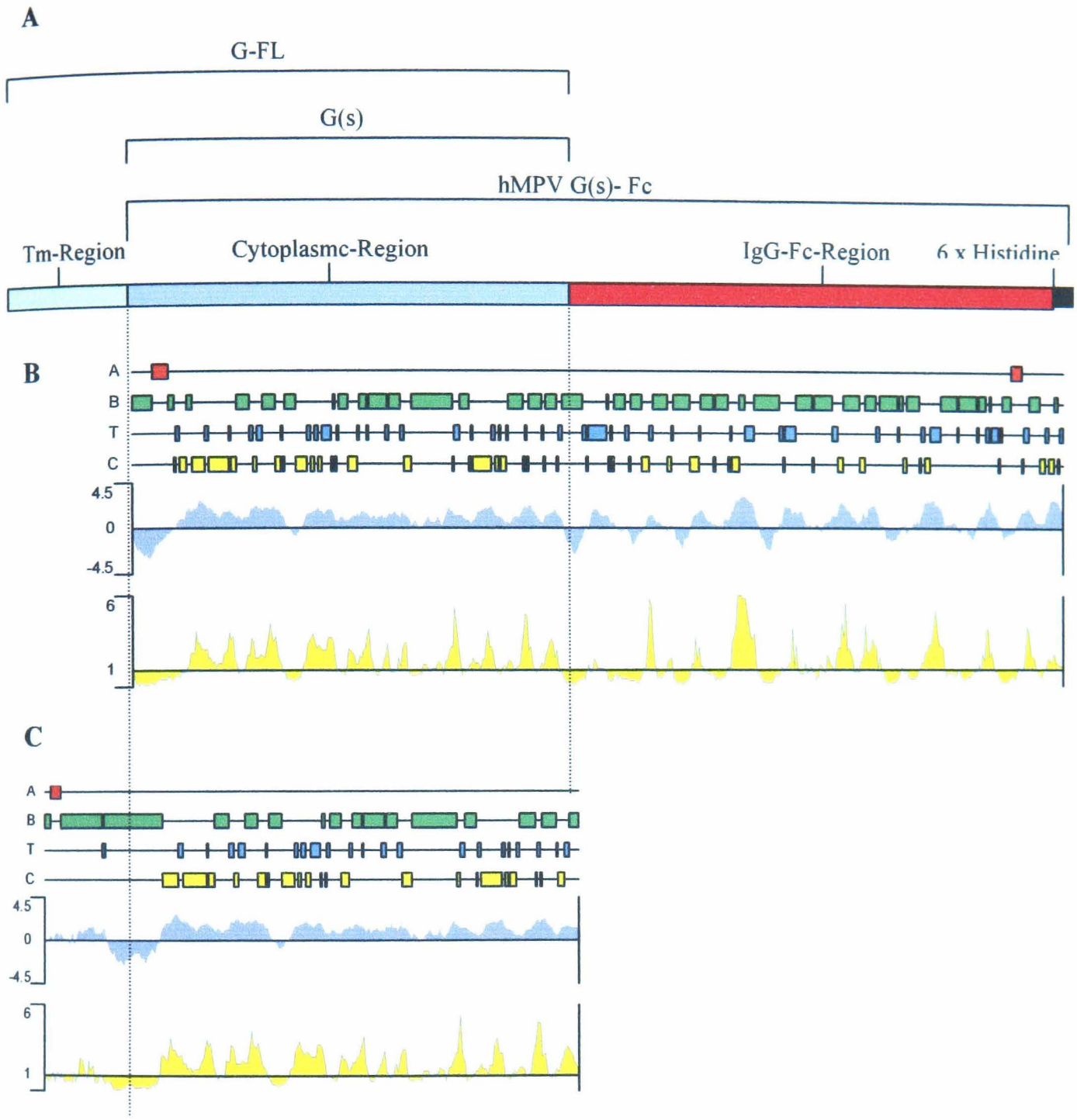


Figure 7.6: Secondary structure protein prediction of the hMPV G (window size of 9 amino acids). (A) Schematic showing the localisation of the IgG-Fc fusion protein and his Tag, (B) Secondary structure prediction of the truncated G protein with the IgG-Fc fusion protein and 6-histidine tag. (C) Secondary structure prediction of the full length G without the fusion protein and tag. α, β, Turn and coil regions were predicted using the Garnier-Robson method (Garnier et al., 1978). Hydrophilicity plot was predicted using the Hydropathy – Kyte-Doolittle method (Kyte & Doolittle, 1982), and the Surface probability predicted using the Emini method (Emini et al., 1985).

7.10.1 hMPV G(s)-Fc baculovirus time course assay

Figure 7.7 shows a time course assay of hMPV G(s)–Fc expressed in High 5 cells. High 5 cells are an insect cell line with a greater capacity for expressing secreted proteins compared to that of SF9 or SF21 insect cells.

As with G-FL there were 2 major bands. The low molecular weight (LMW) band is the predicted size of the non-glycosylated hMPV G(s)-Fc fusion protein while the HMW band may represent an hMPV G(s)-FC glycosylated species although this has not been determined in this study. LMW species may also represent break down products of the hMPV G(s)-Fc protein. Results show that the protein was expressed at 24hpi and consisted of predominantly HMW species. At 48 to 96 hours pi equivalent amounts of the HMW and LMW species were produced. At 96 hours pi the size of the HMW species was slightly reduced, possibly indicating a reduction in the rate of posttranslational modifications. The amount of the LMW species increased over the 4 days. The optimal time for harvesting the expressed protein was therefore 72 hours pi.

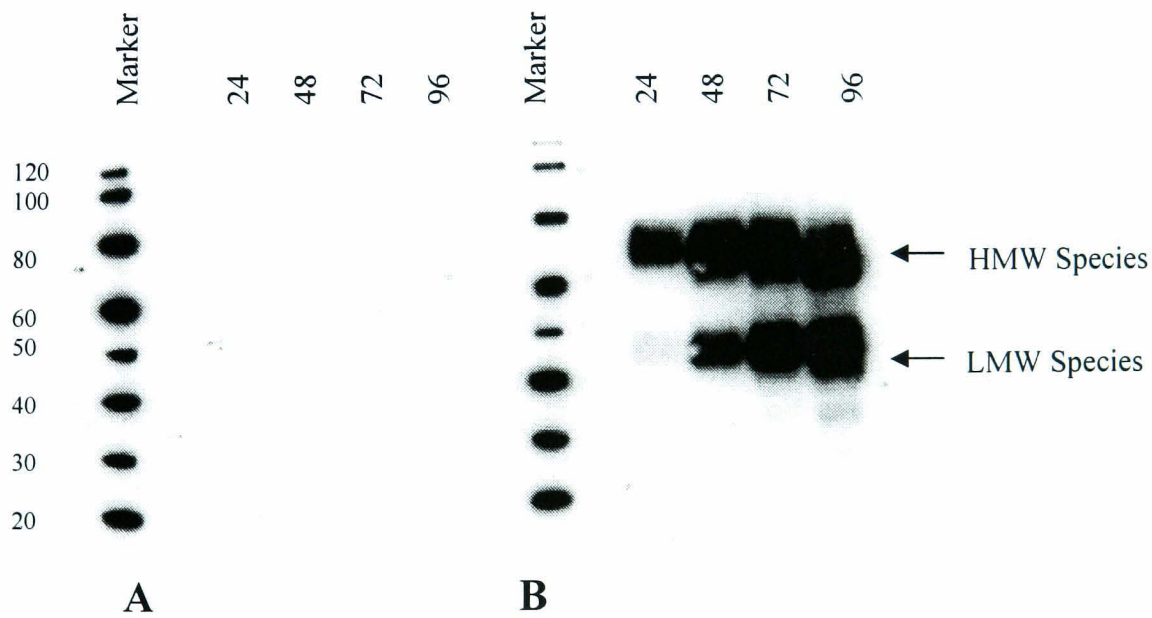


Figure 7.7: Time course assay of hMPV G(s)-Fc expression. Western Blot analysis of hMPV G(s) supernatant taken from HIGH 5 cells :(A) uninfected (B) infected with 5 MOI of the recombinant baculovirus hMPV G(s)- Fc every 24 hours over 5 days. The expressed protein was detected using Rabbit α-Human IgG-Fc 1:1000, and Goat α-Rabbit IgG HRP conjugate 1:2500 followed by ECL reagent and autoradiography.

7.10.2 Purification of G(s)-Fc

Purification of hMPV G(s)-Fc could be achieved through the use of protein A affinity chromatography making use of the inherent properties of the Fc fusion protein, or via the Ni NTA affinity chromatography system used previously for the purification of N in Chapter 6. To assess the binding capacity of hMPV G(s)-Fc to protein A, 96 well plates were coated with supernatant from high 5 cells infected or uninfected with recombinant baculovirus hMPV G(s)-Fc. A 5 fold dilution series of protein A HRP conjugate was applied followed by TMB substrate. The OD was read at 450nm (Figure 7.8).

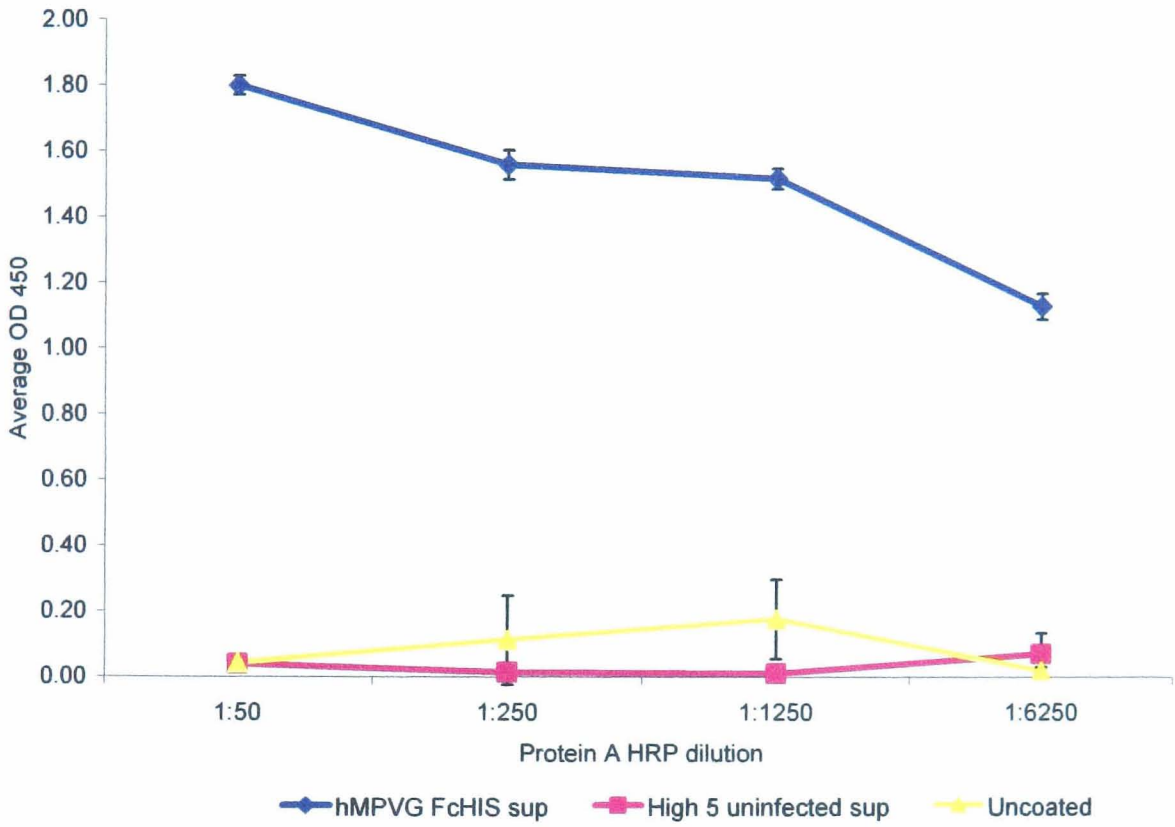


Figure 7.8: Analysis of hMPV G(s)-Fc binding to protein A. A 96 well plate was coated with 100µl of recombinant Baculovirus hMPV G(s)-Fc High 5 infected / uninfected supernatant diluted 1:100 with 10mM PBS and incubated at 37^0C for 30 minutes. Plates were blocked with 5% milk for 1 hour at 37^0C. HRP conjugated protein A was applied in a 5 fold dilution series and detected with TMB substrate and the OD450 reading taken, and the negative OD value subtracted from the positive to give the corrected OD value

The results clearly show the binding of the hMPV G(s)-Fc protein to the protein A HRP conjugate. This indicates that the Fc fusion protein has maintained its functionality despite the presence of the hMPV G(s) protein. A protein A affinity chromatography system (hitrap Column) was therefore utilised for purification of the hMPV G(s) –Fc protein (Figure 7.9).

Prior to purification the High 5 cell culture supernatant containing the hMPV G(s)-Fc protein was applied to a buffer exchange column to exchange the cell supernatant for the 20mM sodium phosphate binding buffer (pH7), recommended by the manufacturer and applied to the Protein A column. The column was washed with binding buffer and eluted using 0.1M citric acid pH3. The different fractions were collected and analysed by western blot analysis and detected with Rabbit α-human IgG (1:1000) followed by Goat α-rabbit IgG HRP conjugate.

The results show that no protein was lost in the buffer exchange process (lane 2). However there was a large amount of G(s)-Fc present in the protein A column flow through (lane 4), and in the first wash fraction (lane 5). There was no protein in the following washes or elution fractions (lanes 6-10) suggesting that the protein failed to bind to the protein A column. Analysis of the column flow through revealed that the pH of the protein in the binding buffer was correct and not affected in the buffer exchange process.

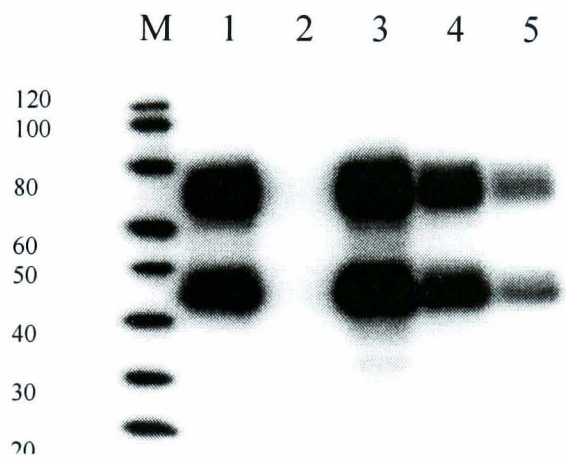


Figure 7.9: Analysis of hMPV G(s)-Fc protein A purification fractions. (A) Western blot analysis with rabbit α-human IgG 1:1000 followed by goat α-rabbit IgG HRP conjugate 1:2500 detected with ECL reagent and auto radiography. hMPV G(s)-Fc high 5 cell culture supernatant was underwent a buffer exchange with 20mM sodium phosphate binding buffer (pH7) and applied to the Protein A column, washed with binding buffer and eluted using 0.1M citric acid pH3. Fractions were collected. (M) Marker, (1) hMPV G(s)-Fc cell supernatant, (2) Column exchange flow through, (3) hMPV G(s)-Fc in binding buffer, (4) protein A high trap column flow through, (5) wash 1, (6) wash 2, (7) wash 3, (8) Elution 1, (9) Elution 2, (10) Elution 3.

Purification of hMPV G(s)-Fc using the Ni-NTA purification system also failed to bind protein efficiently and only small quantities of protein were eluted (data not shown). Following a number of failed attempts at purifying the protein by protein A or Ni-NTA

affinity chromatography under varied conditions, it was decided that hMPV G(s)-Fc antiserum would be generated using the crude cell culture supernatant.

7.6 Generation and Evaluation of hMPV Specific Antiserum.

HMPV specific G(S)-Fc antiserum was raised against the recombinant G(s)-Fc protein and evaluated for its use as potential research or diagnostic reagent (Table 7.6). Antiserum against the hMPV G-FL protein was not generated, as the protein could not be produced in large enough quantities. The rabbit α-hMPV total antiserum discussed in chapter 5 was also evaluated for its reactivity against the recombinant baculovirus expressed G proteins. Both sera were analysed by ELISA, Western Blot, and Immunofluorescence to evaluate their use as potential diagnostic or research reagents.

Table 7.6: hMPV Specific antiserum raised and evaluated in this study.

Antiserum	Immunogen
Rabbit α-hMPV G (s)-Fc	Baculovirus expressed hMPV G(s)-Fc High 5 supernatant 100ng of total protein from the cell supernatant with 10mM Purified LPS 0.5ml incomplete Freund's adjuvant. 4 x 0.25ml of the inoculum was injected at 4 points along the dorsal of the rabbit. 100ng of total protein from the cell supernatant with 10mM LPS and saline was given as a second and third immunogen on days 21 and 40 into the hind leg muscle. Test bleeds taken days 35 and 55. Terminal Bleed taken day 60.

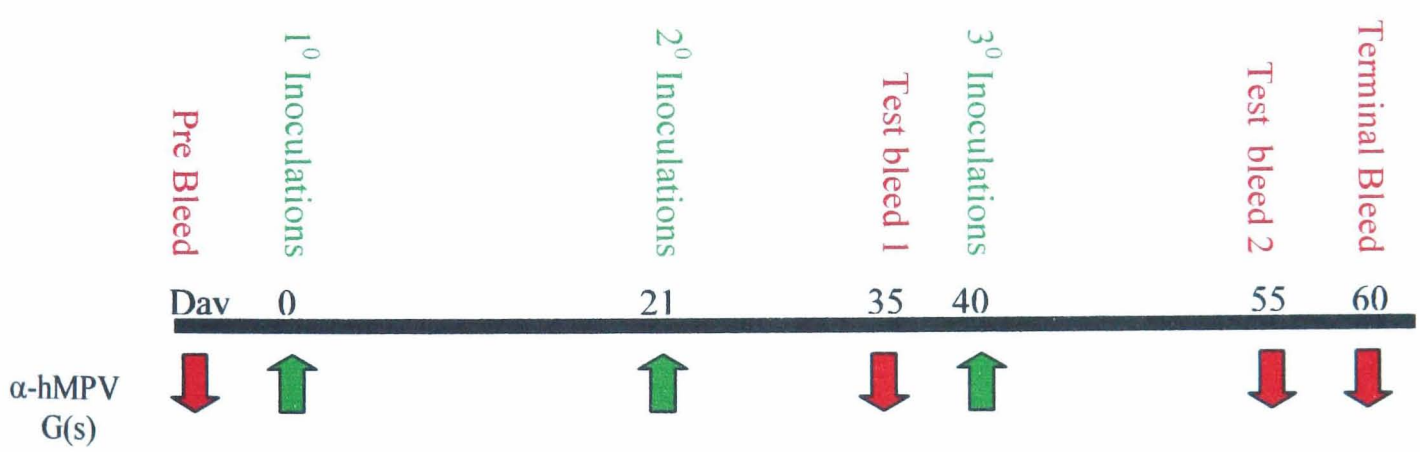


Figure 7.10: Schedule of rabbit inoculations used to generate hMPV specific antiserum. hMPV G(s) Specific polyclonal serum was raised against 100ng of hMPV G(s) Supernatant mixed with purified lipopolysaccharide (LPS- prepared from *Escherichia coli* 0157 [kindly supplied by Dr Henrik Chart at the HPA Colindale]) and incomplete Freund's adjuvant to form an emulsion. Red: rabbit bleeds, Green: inoculation.

7.10.1 Analysis of hMPV Specific Sera against recombinant hMPV proteins by ELISA

The rabbit α-hMPV G(s)-Fc and rabbit α- hMPV total serum were tested by ELISA in a dilution series against the baculovirus expressed hMPV G (S)-Fc protein using culture supernatant from recombinant baculovirus hMPV G(s)-Fc infected and uninfected high 5 cells (Figure 7.11).

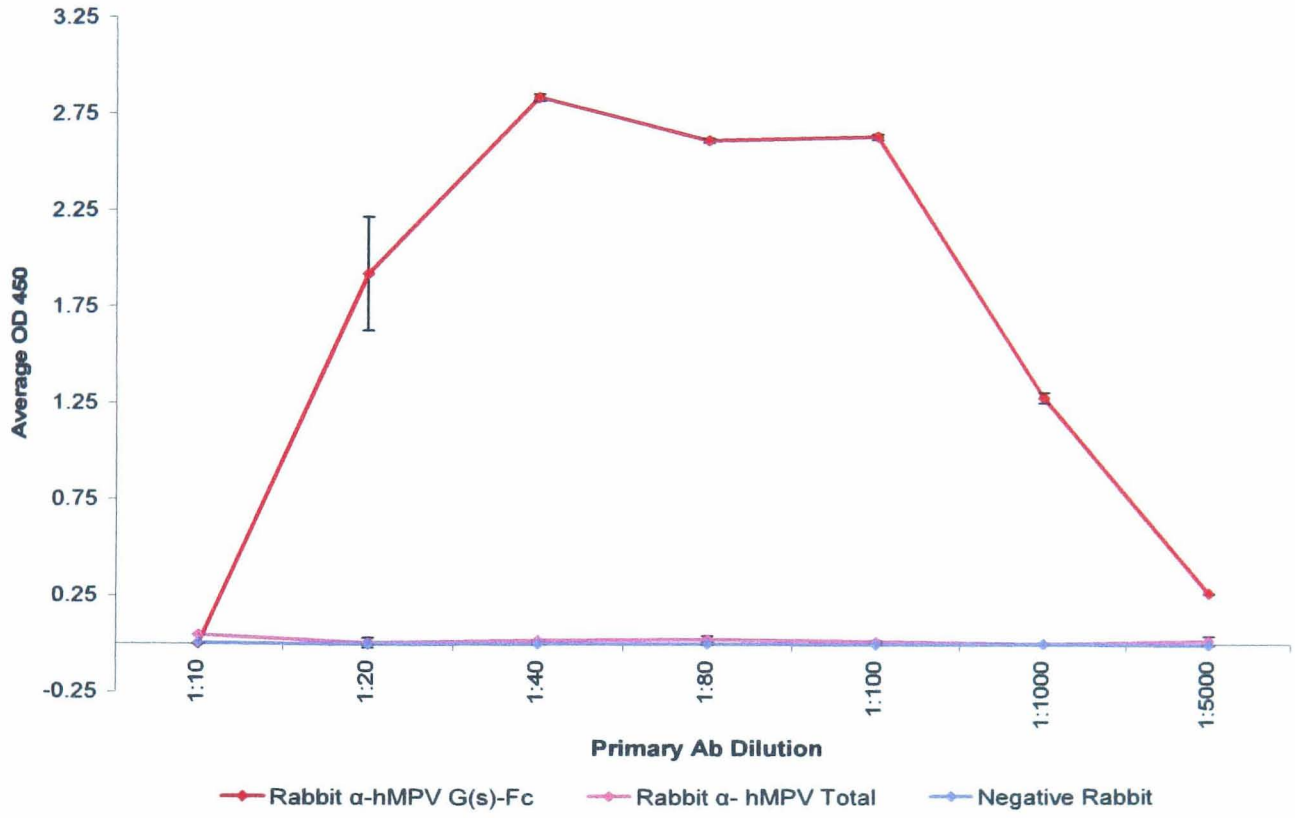


Figure 7.11: Analysis of hMPV specific rabbit sera by indirect ELISA hMPV G(s)-Fc. Error bars show +/- 1 Standard deviation. 96 well plates were coated with approximately 1μg of Rabbit α-human IgG antibody and blocked with 5% milk. HMPV G (S)-Fc positive and negative supernatant (0.8μg of total protein) diluted 1:100 with 10mM PBS and incubated at 37^0C for 30 minutes, and plates washed with 0.1% Tween 20 in PBS. The hMPV specific Rabbit sera were tested in a dilution series from 1:10 to 1:5000 followed by Goat α-Rabbit IgG HRP conjugate 1:2500 and detected using TMB substrate. The absorbance was measured at 420nm reference 620nm, and the uninfected cell culture supernatant OD value was subtracted from that of the infected to give the corrected OD values.

Results showed that the rabbit α-hMPV G(s)-Fc reacted strongly against the G(s)-Fc protein when used between 1:20 and 1:1000 dilutions. The rabbit α-hMPV total antiserum however did not react at all, even at the lowest dilutions tested. The negative rabbit serum was negative at all dilutions tested.

7.10.2 Western blot analysis of hMPV specific antiserum reactivity against recombinant baculovirus expressed hMPV G (S)-Fc.

Rabbit α-hMPV G(s)-Fc and rabbit α- hMPV total at 1:50 and 1:100 dilutions respectively) were tested against baculovirus expressed hMPV G(s)-Fc antigen by Western blot, as shown in Figure 7.12.

Results show that the Rabbit α- hMPV G(s)-Fc antiserum recognises both the high and low molecular weight species at 70 and 50Kda respectively which corresponds with the MW species detected with the positive control antiserum; rabbit α-Human IgG-Fc (Figure 7.11D). The rabbit α-hMPV total antiserum also reacted against both the HMW and LMW species, although at much lower levels, and after a longer exposure. The molecular weight species recognised by each serum is summarised in Table 7.7.

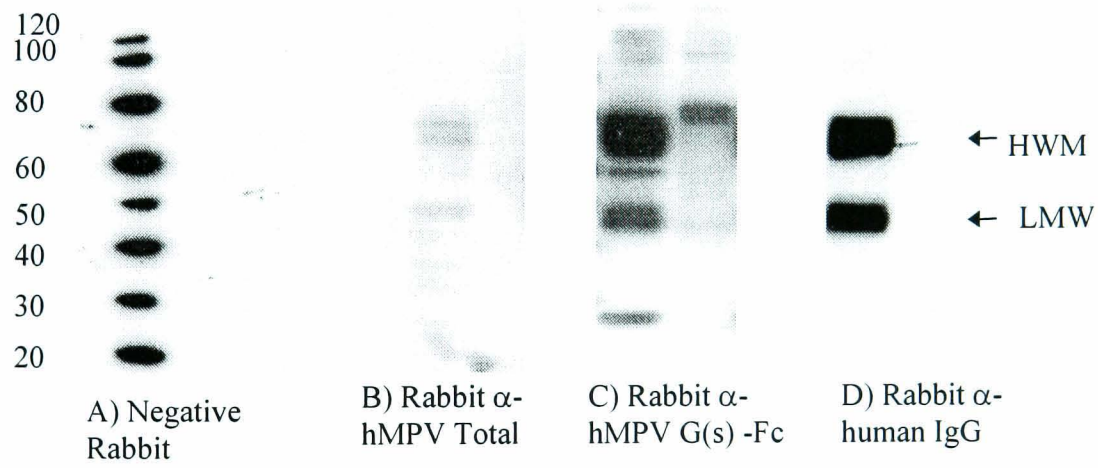


Figure 7.12: Western Blot analysis of hMPV specific Rabbit serum against. (M) Marker, (+) Infected hMPV G(s)-Fc High 5 supernatant, (-) uninfected High 5 Supernatant 2dpi. (A) negative rabbit sera (1:50), (B) rabbit α- hMPV total (1:50), (C) Rabbit α-hMPV G(s)-Fc (1:100), (D) positive control serum against Fc fusion protein: rabbit α-Human IgG-Fc (1:1000). Primary antibody was detected with goat α-rabbit IgG HRP conjugate at 1:2500 followed by ECL and autoradiography.

Table 7.7: Summary of molecular weight profile of the major species recognised by rabbit serum by Western Blot Analysis (KDa)..

Antigen	Rabbit α-human IgG	Rabbit α-hMPV Total	Rabbit α-G(s)-Fc	Negative Rabbit
HMPV G-Fc Supernatant	50 and 70 KDa	50 and 70 (doublet) KDa	28, 50, 60 and 70 KDa	Negative
Uninfected supernatant	Negative	Negative	80KDa	Negative

7.10.3 Analysis of hMPV Specific Sera by IF against recombinant hMPV G-FL and hMPV G(s)-Fc.

The rabbit sera was also tested against the recombinant baculovirus expressed hMPV proteins G-FL and G(s)-Fc by immunofluorescence of recombinant baculovirus infected/ uninfected SF9 cells grown on cover slips (Figure 7.13).

Results show a good level of recombinant protein expression for both G-FL and G(s)-Fc using the Mouse α-6- Histidine MAb (Figure 7.13A). Both Rabbit α-hMPV G(s)-Fc (Figure 7.13C and Rabbit α-hMPV Total (Figure 7.13B) react specifically against both the secreted and full-length G proteins. Rabbit α-hMPV G(s)-Fc did however show some level of cross reactivity with uninfected SF9 cells, was removed by cross absorption.

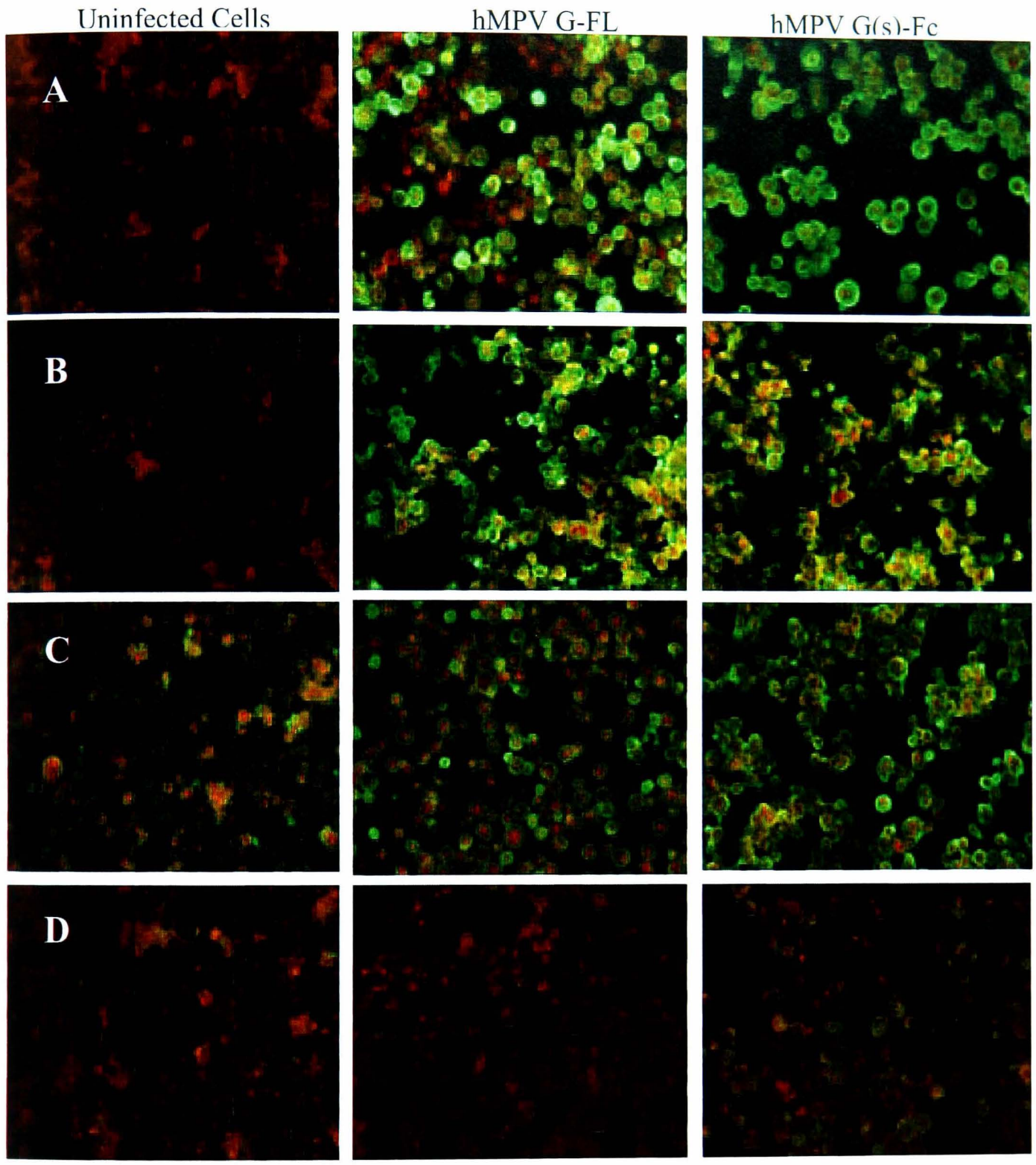


Figure 7.13: Indirect Immunofluorescence of baculovirus hMPV G or Baculovirus hMPV G(s)-Fc infected or uninfected SF9 Cells with (A) Mouse α-6- Histidine MAb 1:50, (B) Rabbit α-hMPV Total 1:25, (C) Rabbit anti hMPV G(s)-Fc 1:50, and (D) Negative Rabbit 1:25 detected using Swine α-Rabbit FITC conjugate 1:40. Cells were counterstained with Evans Blue and visualised by fluorescence microscopy.

7.10.4 The Analysis of hMPV G(s)-Fc specific serum against hMPV infected and uninfected cell lysate.

To further assess the reactivity of the hMPV G specific antiserum, it was tested by Western blot against 2 different hMPV strains isolated from the 2 Northern Ireland clinical samples (Table 7.3) previously standardised by cell equivalence to 1.5 x 10^5 cells/lane on an SDS PAGE gel (Figure 7.14).

Against hMPV virus the rabbit α-hMPV G(s)-Fc was unreactive with the exception of a single band at <20KDa in lanes 2 and 3, also recognised by the negative rabbit serum lanes 2 and 3 suggesting this is likely to be non-specific binding. The rabbit α-hMPV total and negative rabbit reactivates were discussed previously in chapter 5, but were included here as controls.

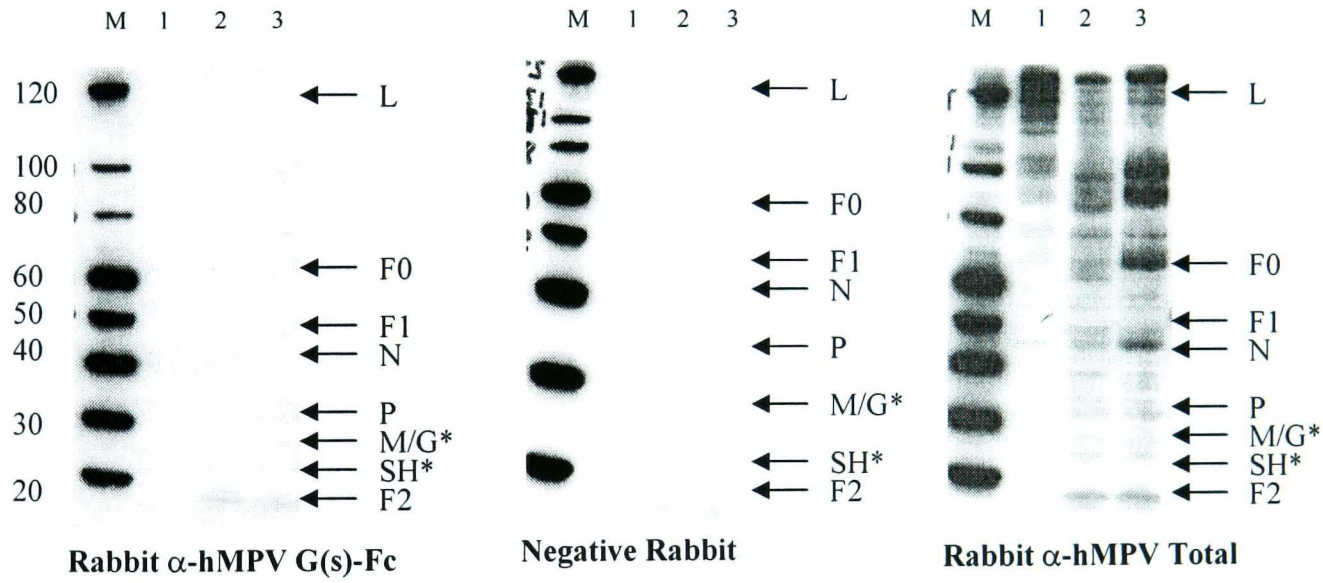


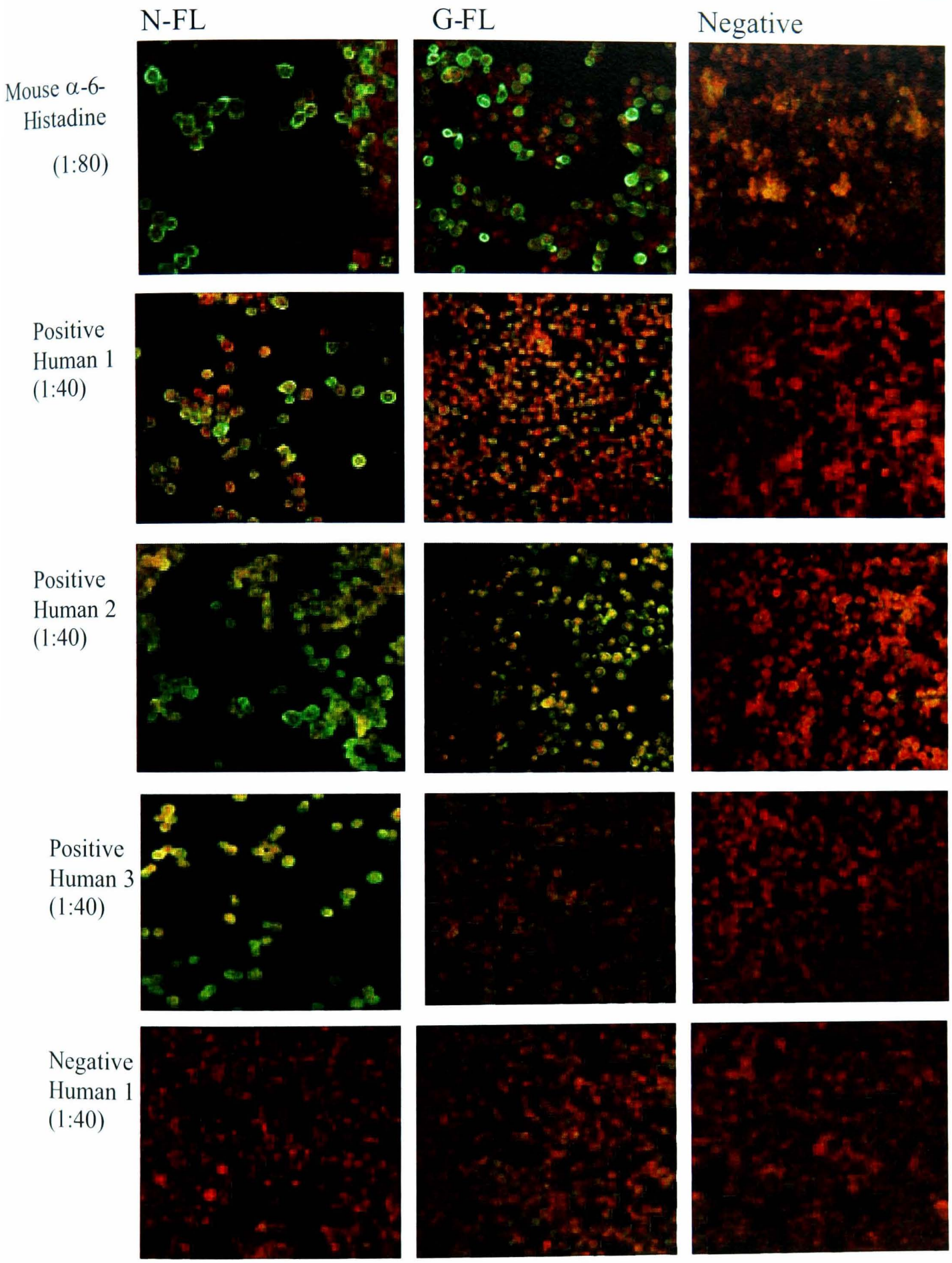
Figure 7.14: Western Blot analysis of various hMPV specific sera against recombinant hMPV proteins and hMPV infected cell lysate. (1) Uninfected LLC-MK2 Cell Lysate, (2) hMPV Cell Lysate -1, (3) hMPV Cell Lysate -2. (*) size of un-glycosylated species. Rabbit α-hMPV G(s)-Fc (1:100), negative rabbit (1:50), rabbit α- hMPV Total (1:50). Primary antibody was detected with goat α-rabbit IgG HRP conjugate at 1:2500 followed by ECL and autoradiography. The virus strains used are detailed in Table 7.3.

7.7 Analysis of human antibody reactivity by indirect IF against recombinant baculovirus expressed hMPV proteins.

As shown in chapter 6 human antibody reactivity against the baculovirus expressed hMPV N protein could be measured by ELISA and western blot analysis. Using the recombinant baculovirus expressed hMPV N protein as a positive control antigen the reactivity of a panel of human sera with known antibody status against N was tested against the recombinant hMPV G-FL protein by IF (Figure 7.15).

Results show that the 3 sera identified as being strongly positive for hMPV N IgG by ELISA are also reactive against the N protein by IF. The serum identified as being negative by the hMPV N ELISA was also negative for the N protein by IF. In response to the hMPV G-FL antigen only serum number 2 appeared to give a convincingly positive result, while serum number 1 showed a very low level of fluorescence. Serum number 3 and the negative serum were both completely negative at 1:40 (and 1:20 dilution; data not shown).

Figure 7.15: Indirect immunofluorescence analysis of a panel of hMPV N positive and negative serum against the hMPV N and full length attachment glycoprotein expressed in the recombinant baculovirus system. SF9 cells were grown on glass cover slips and infected with either recombinant baculovirus hMPV N-FL or recombinant baculovirus hMPV G-FL at an MOI of 3 and 20 respectively. The MOI was pre-determined to ensure relatively equal levels of recombinant protein expression in terms of the number of fluorescing cells detected by the mouse α-6- histidine MAb (data not shown). At 3 days post infection cells were fixed with acetone and immuno-stained with human sera (1: 40) followed by mouse α-human IgG FITC conjugated antibody (1:40) and analysed by fluorescence microscopy. Mouse α-6- histidine MAb (1:50) was used as a positive control and detected using rabbit α-mouse IgG FITC conjugate (1:40). See Table 7.5 for details regarding the human serum.


N-FL
G-FL
Negative
Mouse α-6-Histadine (1:80)
Positive Human 1 (1:40)
Positive Human 2 (1:40)
Positive Human 3 (1:40)
Negative Human 1 (1:40)

7.8 Analysis of human antibody reactivity to hMPV G-FL and hMPV G(s)-Fc by ELISA and Western blot.

The reactivity of a larger panel of human sera against the hMPV G-FL, and hMPV G(s)-Fc baculovirus expressed antigen was analysed in a dilution series by ELISA using the optimised conditions for the hMPV N ELISA described previously (Chapter 6). In each case the corresponding negative cell lysate or supernatant was used to subtract background reactivity (Figure 7.16).

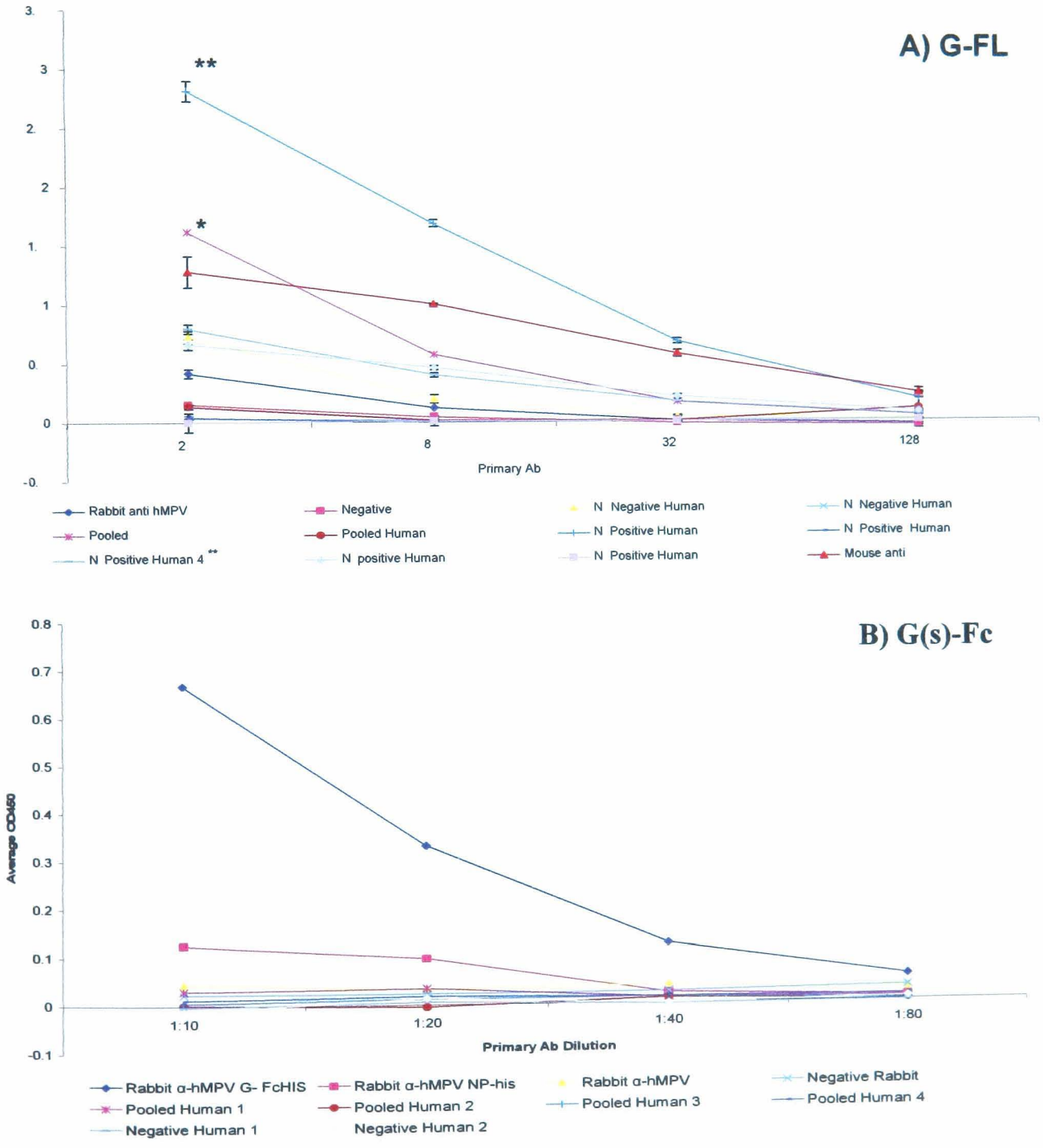


Figure 7.16: Human Ab ELISA reactivity against recombinant hMPV G antigens: (A) hMPV G-FL and (B) hMPV G(s)-Fc. Antiserum was tested in a 2 fold dilution series from 1:10 to 1:80 against the positive and negative antigen. Rabbit sera were detected with goat α-rabbit IgG HRP

conjugated antibody (1:2500). Human sera were detected with Rabbit α-human IgG HRP (1:500) in the case of G-FL and corresponding negative antigen. Human sera were detected with rabbit α-human IgG Fab specific HRP conjugated antibody (1:5000) for the G(s)-Fc and corresponding negative antigen. In both cases the OD value for the negative antigen was subtracted from the positive to give the corrected OD value. Each dilution was tested in duplicate. The corrected mean OD450 values were plotted against the primary Ab dilution. Error bars indicate +/- 1 standard deviation.

Results show that two human sera were reactive with hMPV G-FL, one corresponding with the human sera that was also reactive by IF, and the second a pooled adult human serum (components unknown), all the other sera were non – reactive or only yielded OD450 results which were the same as a human hMPV N negative serum indicated by the arrow. There was no human sera reactivity against the hMPV G(s)-Fc antigen by ELISA (Figure 7.16B). Analysis of a selection of these sera at a 1:75 dilution against the G-FL SF9 cell lysate by western blot analysis is shown in Figure 7.17. The reactivity of the rabbit α-hMPV Total antiserum was also analysed.

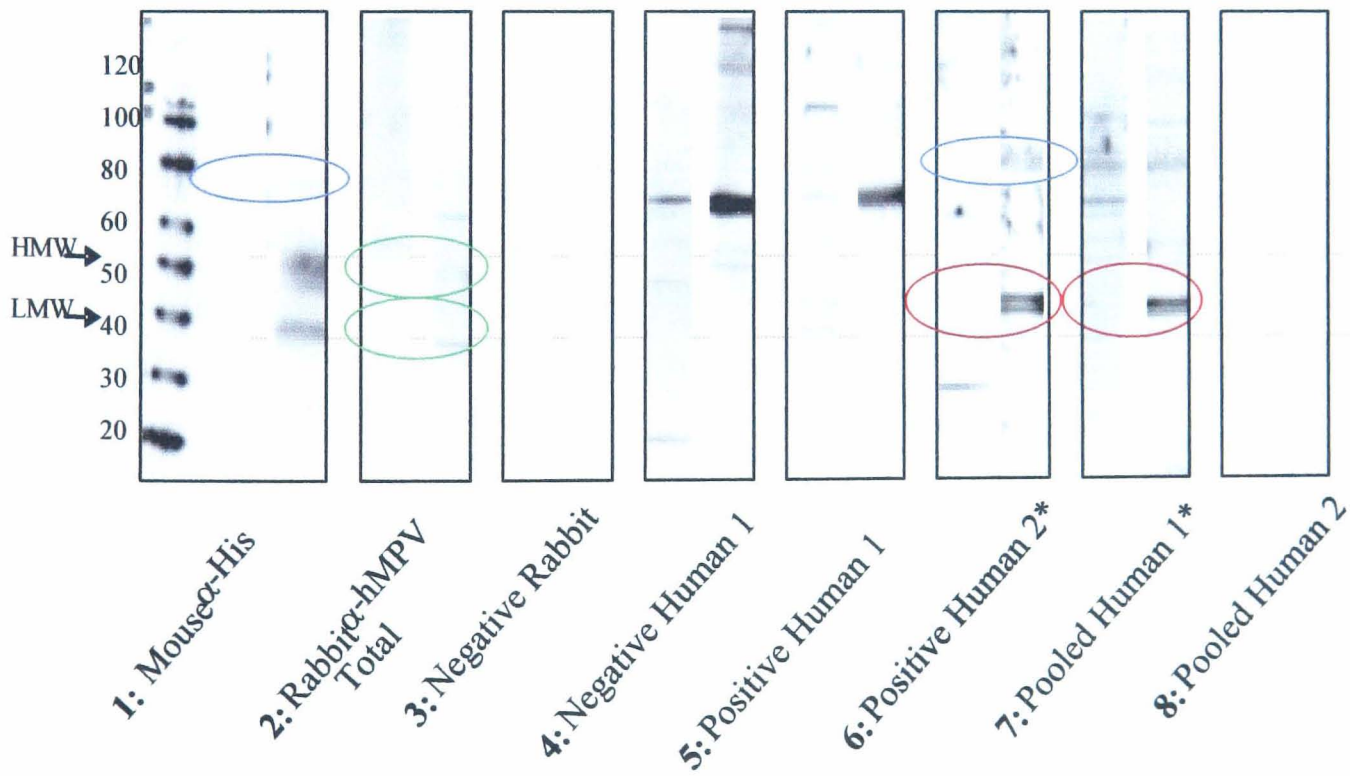


Figure 7.17: Western Blot analysis of human Ab reactivity to hMPV G-FL expressed in the Recombinant Baculovirus system. Human Sera were tested against hMPV G-FL negative (-) or positive (+) cell lysate and analysed with (1) mouse α-6-histidine MAb 1:1000, (2) Rabbit α-hMPV Total 1:50, (3) Negative Rabbit 1:50, (4) Negative Human 1:75, (5) Positive Human 1 1:75 (6)Positive Human 2 1:75 (7)Positive Pooled Human 1 1:75 (8)Positive Pooled Human 2 1:75. Human sera were detected with Mouse α-Human IgG HRP conjugate at 1:10000. The control sera

were detected as described previously. Blots were exposed to ECL substrate and autoradiography.(*) serum reactive against G-FL by IF and ELISA.

Previously shown to be reactive against G(s)-Fc by Western blot and IF and against G-FL by IF, the rabbit α-hMPV Total serum was negative against both G-FL and G(s)-Fc by ELISA. Figure 7.17 show this serum recognises both the HMW and LMW species of hMPV G-FL, although only faint bands are visible (circled in green) and the LMW species appears to be a slightly smaller size. There was no reactivity observed with the negative rabbit serum.

The human antiserum analysed show a number of different MW species that are recognised, most likely cross reactivity with cellular proteins. The two sera (track*6 and 7) which were reactive against hMPV G-FL by ELISA and IF appear also to recognise a species in the hMPV G-FL positive cell lysate that is slightly larger than the correct size for the LMW species of HMPVG-FL (circled in red). The 50Kda ML glycosylated species is less well recognised which may be due to interference of Ab recognition by glycosylation, or differences in the transfer of highly glycosylated species. The human serum in track 6 also recognises a band at approximately 80KDa, circled in blue, which may be non specific binding, which corresponds with a very faint band recognised by mouse α-6 histidine MAb also circled in blue and may represent a species of G –FL with a greater level of O-linked glycosylation, however is this is the case, why is the 50KDa species not recognised?

7.9 Transient Expression of hMPV Attachment Glycoprotein in Mammalian cells

The differences in the posttranslational modifications between insect and mammalian cells may account for the lack of reactivity of human Ab against the baculovirus expressed hMPV G protein. The expression in a mammalian system will yield proteins that more closely represent those expressed during viral infection. Presented here is the transient expression of the hMPV G-FL protein to determine the ability of the protein to be expressed in mammalian cells, with the view to establishing continually expressing cell lines for the analysis of human Ab reactivity, cellular localisation and protein trafficking.

The hMPV G-FL pTriEx vector was transfected into 293T and Vero cells (See section 2.10). At 24hours post transfection the cells were fixed with 4% Paraformaldehyde and permeablised with 0.25% triton X-100. Protein expression was visualised by indirect IF using the mouse α-6-histidine MAb followed by rabbit α-mouse IgG FITC conjugated Ab. The nucleus was stained with 0.1ug/ml DAPI Stain. The stained cells were visualised by fluorescence microscopy.

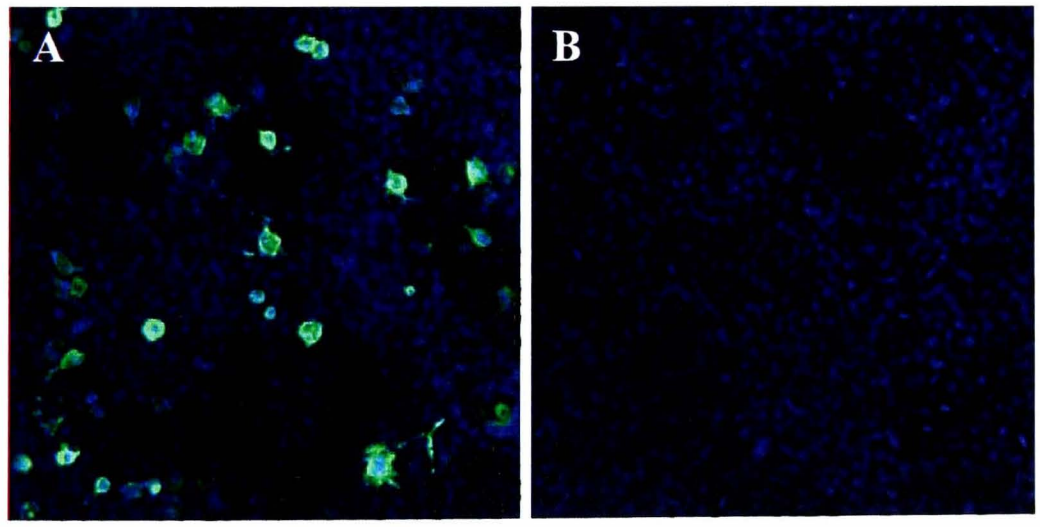


Figure 7.18: Different transfection efficiencies in (A) 293T cells and (B) Vero cells transiently transfected with hMPV G pTriEx 24hours post transfection. The expressed protein was visualised using a mouse α 6-histidine MAb followed by Rabbit α-mouse IgG FITC conjugate. The nucleus was stained with DAPI.

The results show that the hMPV attachment glycoprotein was expressed in 293T cells, but not in Vero cells. 293T cells were therefore used for further expression work. They were however problematic in their own way, attaching loosely to the slide, with a tendency of rounding up. Therefore optimisation of the techniques and transfection conditions was

performed to increase cell viability and transfection efficiency (Data not shown). This included comparing glass versus perminox slides, different cell seeding densities, and different DNA: lipofectamine ratios in the transfection mix.

To assess the localisation of hMPV G-FL in transfected cells at different time points, cells were analysed by IF at 24, 48 and 72 hours post transfection (Figure 7.19).

The results show that hMPV G-FL is expressed at detectable levels throughout the time course. At 24 hours post transfection the protein concentrated around the nuclear envelope, perhaps due to processing through the rough endoplasmic reticulum. At 48 hours post transfection the protein is detected throughout the cytoplasm, and is found in concentrated spots, at 72 hours post transfection the protein appears to be concentrated at the cell surface. It is worth noting however that this analysis was performed with a standard fluorescence microscope, and con-focal microscopy would give much more accurate information as to the localisation of the protein.

IF analysis of transiently transfected cells expressing G-FL was carried out with rabbit and human sera known to be positive or negative for hMPV N IgG, all sera however yielded negative results (data not shown).

Unfortunately due to time constraints the use of this vector for establishing constitutively expressing protein, and further analysis of Ab reactivity against the transfected G-FL protein could not be pursued.

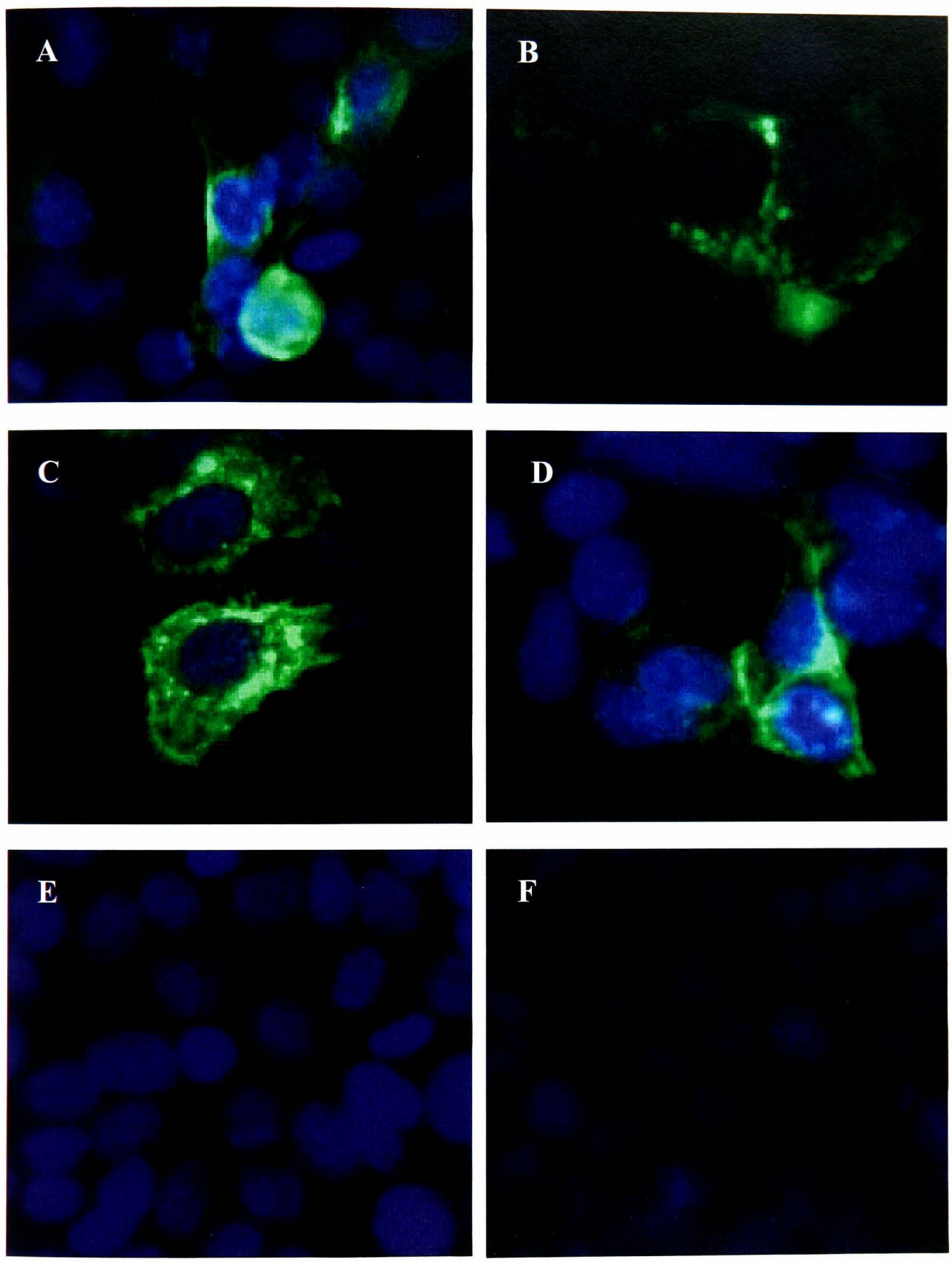


Figure 7.19:hMPV G-FL transiently transfected 293T cells over time. The expressed protein was visualised using a mouse α 6-histidine MAb followed by α-mouse IgG FITC conjugate. The nucleus was stained with DAPI. (A) 24-hours post transfection, (B) 48-hours post transfection, (C) 48-hours post transfection, (D) 72-hours post transfection, (E) pTriEx empty vector 48-hours post transfection, (F) Cells Only at 48-hours

7.10 Discussion

Reported here is the use of the recombinant baculovirus system to express the hMPV attachment glycoprotein and the subsequent generation and evaluation of hMPV G specific antiserum required for the development of serological assays to measure human antibody reactivity against G. This is followed by preliminary work demonstrating the ability to express G in a mammalian expression system.

7.10.1 Recombinant baculovirus expression of hMPV G-FL

The full-length attachment glycoprotein of hMPV was successfully expressed in the recombinant baculovirus system; however, a high MOI of recombinant baculovirus was required in order to achieve detectable levels of protein expression, making the large scale synthesis of protein required for antibody production impractical without successful baculovirus virus purification. The reasons for the low level of protein expression were not investigated within the scope of this work. However protein toxicity, complex post translational modifications, protein instability or degradation, or the need for chaperone proteins (e.g. co-expression with F and SH) may be involved.

The hydrophilicity profile of hMPV G presented in chapter 4 of this thesis, and its similarity with that of hRSV and APV indicates that hMPV G-FL is likely to be a type two transmembrane protein. Whilst problematic in terms of protein extraction and purification the difficulties initially experience with extracting G-FL from the cellular membrane material provides good evidence that the protein was targeted correctly within the cell to membrane organelles such as the endoplasmic reticulum, Golgi apparatus or plasma membrane. The localization of G-FL in insect cells was not investigated further but could be addressed in future work though the use of confocal microscopy using dual labelling techniques.

Different detergents were investigated for their ability to solubilise G, and the findings may be useful for any future work where expression levels of hMPV G-FL are investigated or optimised and subsequent protein extraction and purification techniques are employed.

Whilst ionic detergents such as sodium dodecyl sulphate (SDS) are extremely effective at solubilising membrane proteins, they are also denaturing. Since the ultimate aim of this

work and possible future work will involve the use of G as an antigen in immunoassays and antibody production or in structural studies the use of denatured protein would not be appropriate. Re-folding or renaturation of the protein may be possible by transferring it into a renaturing (non-ionic) detergent or lipid environment, this however is not suitable for all proteins and the removal of SDS can often lead to irreversible aggregation or precipitation of the protein. This technique however has been used successfully for the purification of a chimeric RSV GF protein expressed in the recombinant baculovirus system and analysis with a panel of monoclonal antibodies showed reactivity against both linear and conformational epitopes (Wells et al., 1994).

Non-ionic detergents are mild and non-denaturing as they break up lipid-lipid and lipid-protein interactions rather than protein-protein interactions and are therefore used for isolation of membrane proteins in their biologically active form. However it is important to be aware that non-ionic detergents with short chains (C7-C10) may often lead to inactivation of protein function and therefore non ionic detergents with intermediate length (C14) or longer side chains should be used preferentially (Seddon et al., 2004). A number of non-ionic detergents with long chains were therefore tested and Nonidet P-40 (NP-40) found to be more efficient than Triton-X-100 and Tween20, which also solubilised G-FL although to a much lesser extent. Extraction with NP-40 and Triton-X-100 however resulted in a high degree of molecular weight heterogeneity compared to that seen in membrane fractions and may be a result of increased protein degradation.

Detergents solubilise membrane proteins by mimicking the natural lipid bilayer in which the protein is located. The ability of different detergents to solubilise membrane or transmembrane proteins is dependent on a range of variables including the critical micelle concentration of the detergent; temperature, pH, and the components of the target membrane.

Membranes typically exist in a fluid state of loosely packed lipids, however it is well recognised that distinct membrane domains differing in composition, physical properties and biological functions also exist. This is due to certain lipids which have a greater propensity to pack together more tightly forming what is known as lipid rafts. Lipid rafts have been associated with signal transduction and protein sorting and are often more resistant to disruption with detergents (e.g. Triton-X-100) (Schuck et al., 2003). The association of the G, F and SH proteins of hRSV with lipid rafts has been well

documented, and it is assumed the homologous proteins in hMPV will also be associated with lipid rafts (Henderson et al., 2002, Rixon et al., 2004). Whether G alone (i.e. without F or SH) or indeed whether G in a recombinant system has the ability to associate with lipid rafts is unknown. The ability to solubilise G may therefore depend on the ability to disrupt these membrane domains. The association of G with lipid rafts can be analysed using IF, and electron microscopy.

7.10.2 Recombinant baculovirus expression of hMPV G(s)-Fc

In contrast; the expression of hMPV G(s)-Fc was much greater than that of G-FL. hMPV G(s)-Fc lacks the N-terminal transmembrane and cytoplasmic regions of the protein and is fused to the human IgG Fc region at the carboxyl terminal. The Fc fusion protein has been shown to aid in the expression of secreted proteins by facilitating proper processing and increased solubility. Bioinformatic analysis of the predicted secondary structure, hydrophilicity and surface probability revealed no detrimental effect of the addition of Fc to G(s).

The hMPV G(s)-Fc protein was expressed and secreted into the supernatant of High 5 cells at very high levels when grown in suspension. Purification of G(s)-Fc using protein A columns however was not successful despite demonstrating the ability of the protein to bind protein A in an ELISA. This is likely to be due binding buffer conditions or to steric hindrance within the column.

The hMPV G(s)-Fc supernatant was nonetheless used to raise an hMPV G specific polyclonal antiserum which was then evaluated as a possible research or diagnostic tool.

7.10.3 Molecular weight analysis and glycosylation

Western blot analysis of both G-FL and G (S)-Fc expressed in the recombinant baculovirus system yielded 2 distinct bands of approximately 33 KDa and a smear from 40 to 55 KDa with the major species at 50 KDa for G-FL (Figure 7.4), and two major species of 45 and 70 KDa for G(s)-Fc (Figure 7.7). The low molecular weight band for each recombinant protein represents the expected molecular weights for the proteins without post translational modification. The HMW bands are likely therefore to represent species that have undergone post translational modification the most likely glycosylation. The data presented in chapter 4 demonstrates that hMPV G has a high potential for O-linked

glycosylation, particularly in the extra cellular domain of the protein. The G-FL protein used for the expression work presented here contains 37 serine residues and 39 threonine residues which have the potential to be glycosylated with O-linked carbohydrates. Furthermore this protein contains 2 potential N-linked glycosylation sites which if glycosylated will give a predicted MW species of approximately 39-40 kDa for which a band is visible (Figure 7.4). Although the presence of glycosylation was not investigated within the scope of this work there has much work already published on the presence of glycosylation in the G proteins of hRSV and other pneumoviruses, in virally infected cells and in the recombinant baculovirus expression system. The pattern of molecular weight distribution seen in this work is consistent with that observed for the G proteins of other pneumoviruses and indeed hMPV.

Expression of hMPV G in the baculovirus system has also been reported by Bastien et al (2004) (Bastien et al., 2004). In that study the G protein from an A and B isolate was expressed yielding molecular weights of 38 to 80 KDa, with a major band at 50 KDa for the A subtype protein and a smear of 45 to 75 KDa, with the dominant species migrating at 45 and 58 KDa for the B subtype protein. These observations correspond with the observed molecular weights for G-FL in this study, with slight differences being accounted for by the presence of the histidine tag used in this work and potential differences in the level of glycosylation due to heterogeneity in the number of potentially N- and O-linked glycosylation sites between different strains and potential glycosylation differences of the different cell lines used (SF9 cells in this study and SF21 cells used by Bastien et al 2004). Interestingly Bastien et al did not appear to observe any non-postranslationally modified protein (26 KDa) as observed in this study (33 KDa) but this may purely be due to relative levels of the different species.

To investigate the presence and nature of glycosylation; glycosylation inhibitors such a tunicamycin and monensin, which inhibit N- and O-linked glycosylation respectively, could be used in conjunction with western blot analysis or radio labelling to investigate differences in the MW of the spices generated. These techniques have been used successfully by a number of investigators to characterise the glycosylation profiles of a number of glycoprotein's including the RSV G (Collins, 1990, Collins & Mottet, 1992, Wertz et al., 1989).

7.10.4 Transient expression of hMPV G-FL in mammalian cells

The expression of G-FL in transfected mammalian cells was also successful. Over a time course increased levels of protein expression was observed and was optimal at 48 hours post transfection. The expressed protein was localised throughout the cytoplasm of the cell with concentrated areas of fluorescence (punctuate dots) which may represent accumulation in the endoplasmic reticulum and Golgi apparatus. At 48hours post trasnfection G appeared to spread into thin extensions protruding from the cell (Figure 7.19). Confocal microscopy however should be employed to address more accurately the localisation of this protein in the cell.

Expression of G in the mammalian expression system offers a possible advantage to the recombinant baculovirus system expressing proteins that more accurately represent the native protein; particularly in terms of post translational modifications such as glycosylation, which is known to be more limited in insect cells. Furthermore the use of the mammalian expression system to establish constitutively expressing cell lines would be a vital tool for studying the trafficking and localisation of this protein when combined with confocal microscopy and pulse chase experiments.

Sullender and Britt (1996) conducted a study analysing the glycosylational differences of hRSV G expressed in insect cells and in mammalian cells. Their results showed that a greater level of glycosylation was observed in the RSV G protein expressed in mammalian cells. However despite the reduced level of glycosylation there was little effect on monoclonal antibody recognition of the baculovirus expressed protein. Furthermore the baculovirus expressed RSV G protein was used to raise neutralising antibody in cotton rats which protected the rats upon challenge with RSV, indicating that the baculovirus expressed RSV G proteins had antigenic and immunogenic features similar to that of the G protein expressed in mammalian cells.

7.10.5 HMPV specific and human antibody reactivity against hMPV G antigens

Table 7.4 summarises the reactivity of the hMPV G specific polyclonal antiserum raised against hMPV G(s)-Fc (described in this chapter), Rabbit α- hMPV Total raised against an hMPV infected cell lysate (described in Chapter 5), and a panel of human serum with known antibody status against recombinant baculovirus expressed hMPV N protein (Chapter 5 and 6) against the different G antigens described above and hMPV itself.

Table 7.8: Summary of hMPV specific antiserum reactivity against recombinant hMPV G antigens.

		Anti-serum			
Antigen Preparation	**Analysis**	**Mouse α-6-Histidine MAb**	**Rabbit α-hMPV G(s)-Fc**	**Rabbit α-hMPV Total**	**Human antiserum ****
Recombinant baculovirus hMPV G-FL	**ELISA**	++ (1:1000)	ND	- (1:20)	++ (1:20)
	WB	++ (1:1000)	+/- (1:100)	+ (1:50)	++ (1:75)
	IF	+++ (1:50)	+ (1:50)	+ (1:25)	+ (1:40)
Recombinant baculovirus hMPV G(s)-Fc	**ELISA**	+ (1:1000)	++ (1:80)	- (1:20)	- (1:20)
	WB	+ (1:1000)	+++ (1:250)	+ (1:50)	ND
	IF	+++ (1:50)	+++ (1:50)	++ (1:25)	ND
hMPV infected cells	**WB**	NA	- (1:100)	+ (1:50)*	ND
	IF	NA	ND	+ (1:25)*	ND
hMPV G-FL transfected mammalian cells	**IF**	+++ (1:50)	- (1:50)	- (1:25)	ND

Footnotes: WB: Western blot analysis; IF: Immunofluorescence, (-) No antibody reactivity (+) Weak antibody reactivity, (++) moderate antibody reactivity, (+++) strong antibody reactivity. The values given in (brackets) represent the antibody dilution which was tested or yielded a positive result. (NA) not applicable, (ND) not done. (*) See also chapter 5. (**) two human sera only, the remainder were negative.

7.10.5.1 Rabbit α-hMPV G(s)-Fc

The rabbit α-hMPV G(s)-Fc polyclonal antiserum raised against the recombinant baculovirus expressed G(s)-Fc protein, reacted strongly against the homologous protein by Western blot, IF and ELISA. It also recognised recombinant baculovirus expressed hMPV G-FL by IF and possibly by Western blot, although it is worth bearing in mind that both the recombinant proteins contain a 6-histidine tag against which this antiserum may be reacting and repeat analysis should be performed following removal of the tag. Rabbit α-hMPV G(s)-Fc was not reactive against hMPV infected cell lysate by Western blot analysis or hMPV G-FL expressed in the mammalian expression system by IF, but this could be concentration dependent.

Failure to recognise the G protein in hMPV G infected cell lysate, may be due to strain differences, although both belong to the A lineage, or more likely the relative level of hMPV G protein expression in the cell lysate preparation. Due to the position of the G gene in relation to the promoter at the 3' end of the genome, it may possible that the level of G protein expression in hMPV infected cells is below the detection limit. Levels of G protein expression could be monitored over the course of hMPV infection by quantitative RT-PCR targeting mRNA, or by northern blot analysis.

This however does not explain the lack of reactivity with the mammalian cell expressed G-FL protein in which the homologous strain was used. Although the work done was only preliminary and optimisation may yield a different result. This suggests that there are possible differences in the conformational epitopes of the hMPV G(s)-Fc protein against which the antiserum was raised and that of the G-FL protein expressed in the mammalian cells. This antiserum is therefore unlikely to be a useful reagent for further research or diagnostic use except against its homologous hMPV G(s)-Fc protein.

7.10.5.2 Rabbit α-hMPV total

It was demonstrated in chapters 5 and 6 that the rabbit α-hMPV total antiserum contains IgG that was reactive against the hMPV N protein expressed in the recombinant baculovirus system. It was also demonstrated in chapter 5 that this serum reacted specifically against hMPV infected cells by IF and Western blot analysis clearly reacting against proteins with the correct molecular weight for the N, P and F0 proteins (Figure 7.14). Against the recombinant baculovirus expressed hMPV G-FL and G(s)-Fc, this

antiserum was reactive by IF, and Western blot analysis, indicating that either linear or conformational epitopes were recognised. By western blot both un-glycosylated and glycosylated species were recognised although at very low levels suggesting that either the α-G antibody level in this rabbit serum was quite low or there are only a few conserved epitopes in the G proteins of different strains. This does however suggest that at least to some extent the antigenicity of the G protein expressed in the baculovirus system was maintained.

Surprisingly this antiserum failed to detect the hMPV G-FL protein expressed in the mammalian expression system by IF. It is unlikely that strain differences between the virus used to generate the antiserum, and that used in the expression work account for this lack of reactivity as the same strain was used for all expression work and this antiserum clearly recognises the baculovirus expressed G proteins by IF. These however were preliminary data and optimisation of protein expression levels in the mammalian system, fixation techniques and the IF assay itself may yield a different result. This antiserum may provide a useful tool for the analysis of antibody reactivity against hMPV G proteins although assay optimisation is required.

7.10.5.3 Human antibody reactivity.

A panel of human serum known to be either positive or negative for hMPV N IgG was tested against the different hMPV G antigens.

By IF only 1 of the 4 sera positive for hMPV N IgG tested were reactive against the hMPV G-FL protein expressed in the baculovirus system. This same antiserum, plus an additional antiserum pool was also reactive against G-FL, but not G(s)-Fc by ELISA, suggesting there are possible conformational differences between G(s)-Fc, and that of native G and G-FL. Or that the majority of antibody is targeted towards the more conserved transmembrane and cytoplasmic regions of the G protein.

By Western blot these same two sera appeared to be reactive against a species which is slightly larger than the expected size of the non-glycosylated hMPV G-FL protein species, and smaller than the glycosylated species. This is either non specific, or may be reactivity of the sera against a g species without the tag, but was not investigated further due to time constraints. In general however the human sera tested were not reactive against either glycosylated or un-glycosylated species.

Work published by Bastian *et al* (2004) shows the reactivity of polyclonal guinea pig antiserum raised against two different hMPV isolates was analysed by western blot and immuno-precipitation of baculovirus expressed hMPV G proteins from the homologous virus. By western blot analysis both glycosylated and un-glycosylated species were recognised. Immuno-precipitation however demonstrated that this antiserum did not react with highly glycosylated species (Bastien et al., 2004). This observation suggests that antibody to the glycosylated forms of G may be poorly induced, and that glycosylation may contribute to the interference of antibody induction and recognition of the G protein. The presence of glycosylation in the G protein of hRSV G has also been reported to have a similar effect on human antibody reactivity (Palomo et al., 2000). Some hRSV epitopes are dependent on glycosylation, thus, if glycosylation is different in different cell types (even if both mammalian) then the antigenic profile of this protein can be changed (Garcia-Beato et al., 1996).

In further support of this hypothesis Skiadopoulos *et al* have reported; using a recombinant parainfluenza virus (rPIV) expressing the hMPV G protein; that G was only very weakly immunogenic and very little if any neutralising antibody could be detected in serum taken from hamsters infected with the recombinant virus, despite high levels of G protein expression. In contrast; the F gene expressed in the rPIV system was highly immunogenic and protective (Skiadopoulos et al., 2006). This differ significantly to the findings of all other paramyxoviruses in which all the F and G/H/HN proteins examined to date induce protective antibodies (de Swart et al., 2005, Martinez & Melero, 1998, Paterson et al., 1987, Schmidt et al., 2001).

It is clear from the work presented in this chapter that analysis of antibody reactivity to the attachment glycoprotein of hMPV will not be straight forward and a range of techniques need to be employed. It is likely given the large degree of strain diversity shown in chapter 4 that the best results for breaking down antibody reactivity and mapping epitopes will be achieved using a homologous antiserum to the strain being analysed. While it is clear that the human antiserum reacted with the baculovirus expressed hMPV G-FL protein, the hMPV G(s)-Fc protein is unlikely to be of any benefit in further research or diagnostic use, and energies should be focused on improving the expression levels of hMPV G-FL. It does highlight the importance of maintaining the integrity of the protein. As more information is accumulated with respect to the structure and function of this protein demonstrating that

the integrity of this protein in recombinant systems will become easer. However the glycosylation profile, localisation of the protein within the cell, and antibody recognition can all be analysed.

Given that apparent complexity of antibody reactivity against this protein the baculovirus expression system however should be treated with caution and its limitations kept in mind at all times. A mammalian expression system may therefore provide more reliable results. Although human antibody reactivity against the G-FL protein expressed in the mammalian expression system was not investigated within the time available for the completion of this work, the preliminary data presented in this chapter demonstrate that hMPV G can be successfully expressed in the mammalian cell expression system and paves the way for constitutive cell lines expressing the G protein to be established.

It is clear that the G protein of hMPV is not a major immunogen and the amino acid and glycosylation heterogeneity of the protein is likely to contribute to the lack of antibody recognition seen in this work and that of others. Inclusion of G in any potential vaccine is therefore unlikely to yield any benefits.

However data is some what contradictory in the respect that the large degree of amino acid variation observed in this protein (Chapter 4) indicates that the protein may be under positive selection, most likely via immunological pressure (although more temporal data is required to prove this), suggesting that many antibodies targeted to G may indeed be protective in order to drive these amino acid changes, however the data presented in this chapter and that of others suggests otherwise (Skiadopoulos et al., 2006).

Chapter 8

Concluding Remarks

As with all newly emerging or identified viruses it is vital to understand the potential risk of the virus to human and or animal health by characterising any virally associated disease, establishing its epidemiology and pathogenesis, identifying those most at risk of infection, and evaluating whether vaccine and or antiviral drug development should be pursued.

Since the discovery of hMPV by Dutch researchers in 2001, hMPV has been reported world wide, and is considered an important cause of respiratory tract infection in people of all ages, in particular infants, young children, the elderly and immunocompromised populations (Boivin et al., 2003, Hamelin & Boivin, 2005, van den Hoogen et al., 2001, van den Hoogen et al., 2004, van den Hoogen et al., 2003) . At the start of this work in 2002 very little was known about hMPV. The aim of this thesis was to contribute to the overall understanding of hMPV, by assessing its contribution to disease in the UK community, and the diversity of circulating hMPV strains through the development of a number of research and diagnostic tools and reagents.

8.1. Prevalence of hMPV

It has been demonstrated in this work and that of others that hMPV is responsible for a substantial proportion of ARTI in young children, and is a frequent cause of hospitalisation (Chapter 3). Serological evidence suggests that hMPV infections occur throughout infancy, although the majority of primary infections occur in children 16 months or older, with approximately 70% having been infected by the age of 6 years (Chapter 6). This is somewhat older that that seen for hRSV, where a majority of children will have been infected by the age of 2 years (Ebihara et al., 2004), and may in part explain why the severity of disease associated with hMPV infection is generally considered to be milder than that of hRSV. Although not investigated within the scope of this work, the clinical manifestations of hMPV infection in young children appear to be clinically indistinguishable from those with hRSV, with the most common hMPV associated diagnosis being bronchiolitis, pneumonia and bronchitis (Boivin et al., 2003, van den Hoogen et al., 2003).

hMPV is also an important cause of community acquired ARTI in people of all ages, as illustrated by the sizeable number of hMPV infections identified in adults presenting with influenza like illness in this study (Chapter 3). HMPV infection in adults has also been associated with bronchitis, pneumonia, and exacerbations of asthma and COPD (Martinello et al., 2006, Rohde et al., 2005, Williams et al., 2005). The major risk factors appear to be

advancing age, underlying cardiopulmonary or immunocompromised conditions, and COPD. To date a majority of studies investigating hMPV have focused on children, however, it is important that further investigations are continued in order to define the role of hMPV in these adult populations, especially considering the demographic changes that have occurred over the past 30 years which have resulted in an ageing population. Since 1971 to 2004 the percentage of people over the age of 65 years has increased from 13 to 16% with people aged 85 and over rising from 7 to 12%. In contrast, over the same period of time the percentage of people under 16 years of age has fallen from 25 to 19%, and this trend is projected to continue. In addition life expectancy, has risen at a much faster rate than healthy life expectancy (expected number of years in good heath), and the number of years a person can expect to live in poor health has risen from 6.5 to 8.7 years for males and from 10.1 to 11.6 years for females from 1981 to 2001 (ONS, 2005).

Given that people are living longer, and in poor health the significance of hMPV, and other respiratory viruses in this group is likely to increase, and may contribute significantly to the number of elderly with ARTI seeking medical attention. Overall, however hMPV is less frequently associated with disease in adults compared to that of children; nonetheless, asymptomatic infections are rarely detected in either group.

HMPV has a seasonal distribution, peaking during the typical respiratory virus season (winter and spring months in temperate climates, and late spring early summer in subtropical countries). In some studies, including this one, hMPV has also been reported to occur sporadically or circulate throughout the summer, although at a much reduced rate (Chapter 3). Peak circulation occurs at a time when many other common respiratory viruses (including hRSV, influenza, PIV, rhinovirus and coronaviruses) are also circulating, it is not surprising therefore that co-infections of hMPV with one or more of these viruses are often detected, the most common being hRSV (Chapter 3).

Whether co-infection of hMPV is associated with a more severe clinical picture is yet to be established. While some groups have reported little or no difference in the severity of disease one group has reported increased severity of hRSV associated bronchiolitis in hospitalised children also testing positive for hMPV (Greensill et al., 2003, Semple et al., 2005). During the recent SARS coronavirus outbreak in 2003, hMPV was the most commonly detected co-pathogen (Kuiken et al., 2003). In-vivo work in monkeys however demonstrated there was little difference in the severity of disease or the pathological

findings of monkeys infected with either SARS CoV alone or co-infected with SARS CoV and hMPV (Fouchier et al., 2003, Kuiken et al., 2003, Kuiken et al., 2004). This study however was limited to only a very small number of animals and the multifactorial influences on respiratory tract infections should not be underestimated.

It has often been observed that infection with one pathogen may lead to a predisposition to infection or increased severity of disease by another. More favarable environments may arise as a result of co-infection by a number of mechanisms, for example, weakening host immune repossess, enhancing viral replication by providing accessory proteins, or by maintaining cells in a particular stage of cell cycle.

There is at present no in vitro evidence reported on the effect of co-infection in hMPV replication, but this could easily be studied using laboratory adapted strains of hMPV capable of replicating in the same cell lines as other viruses. The effects of individual viral proteins could be studied through the use of constitutively expressing cell lines or chimeric viruses. One such study conducted by Pham *et al* (2005) constructed recombinant hMPV viruses in which either the N or P genes of hMPV where substituted with the homologous genes from APV. Both recombinant viruses replicated more efficiently in Vero cells, but were attenuated in vivo (AGM) when compared to parental hMPV. The authors postulated that the APV N and P proteins may function more efficiently in Vero cells compared to that of hMPV indicating possible interactions with cellular components. Alternatively APV N and P may function more efficiently in context with other hMPV proteins compared to those of hMPV although the in vivo results contradict this hypothesis (Pham et al., 2005). Whether enhanced replication is observed in the presence of hRSV N or P proteins would be interesting and more relevant in terms of addressing questions relating to possible mechanisms for enhanced disease during naturally occurring co-infections with these two viruses.

8.2. Detection

It is clear from the data presented in this thesis that cell culture isolation of hMPV is very difficult and time consuming, and for studies such as the one reported here where the samples are often of poor quality and low viral copy number cell culture isolation is not an appropriate means of detection. Although a recent publication by Ingram *et al* (2006)

demonstrated the successful use of human bronchial epithelial cells (16HBE140) for the isolation of hMPV from clinical samples (Ingram et al., 2006) . The poor growth properties of hMPV nonetheless in part explain why the virus has not been identified until recently.

Genetically modified cell lines may be used to improve the growth properties of hMPV, and may include cells constitutively expressing proteins such as a putative receptor, or as shown above, proteins from closely related viruses such as the APV N or P proteins which enhance replication, or alternatively knockout cells lacking the ability to produce antiviral interferons. One such cell line used this study was a HEP2 cell line which constitutively expressed the V protein from SV5 which antagonises the antiviral interferon pathway by targeting STAT for proteasome degradation. This particular cell line did not yield any isolates (Chapter 3), although it has since been shown that HEP2 cells do not support the growth of laboratory adapted strains of hMPV. An alternative cell line such as LLC-MK2 cells which have been shown to support hMPV growth may therefore yield better results if genetically modified in a similar way. Other factors should also be investigated; for example, do interactions occur between cellular proteins and the components of the RNP? If so what are they, and can they be enhanced or eliminated to improve replication? Alternatively could hMPV replication be cell cycle dependent, and if so at which stages is replication most efficient?

Currently RT-PCR is the most commonly used technique for the detection of hMPV and has proved to be the appropriate assay for the detection of hMPV in this work. It is should be kept in mind that continuous review of the primers used in such assays is vital to ensure all possible strain types are detected.

Immunofluorescence using specific antibodies to detect virus in clinical samples is a frequently used technique for respiratory viruses in many diagnostic laboratories. Two polyclonal antibodies generated in this work against hMPV infected cell lysate and recombinant baculovirus expressed hMPV nucleocapsid protein have been evaluated for their reactivity. Although not yet formally evaluated the rabbit α-hMPV total antiserum has the potential to be used as a reagent for direct IF of clinical material. Although a number of studies have shown that immunofluorescence is not as sensitive as RT-PCR for the detection of hMPV this may nevertheless be a useful approach for diagnostic laboratories.

8.3. Seroprevalence of hMPV

A recombinant baculovirus expressing the hMPV nucleocapsid protein (N) was generated, and proved to be a useful source of antigen for serological assays and the production of polyclonal antiserum (Chapter 5 and 6). This antigen was used successfully in an indirect ELISA format, and in western blot analysis and immunofluorescence. Data gathered using the ELISA to test a panel of age stratified sera, showed that the assay had good level sensitivity and specificity (Chapter 6).

Although not useful as a diagnostic assay during the acute phase of infection, such an approach provides a useful tool for continuing research into the seroprevalence of hMPV in different populations, and may be used during outbreak situations or as a confirmatory test through the analysis of acute and convalescent serum.

The seroprevalence data presented in Chapter 6 highlights the important point that hMPV infections frequently occur despite the presence of pre-existing hMPV specific antibodies and raises a number of questions concerning the acquisition and duration of immunity, and the ability of the virus to evade host immune responses. It is clear from the seroprevalence study that virtually all adults have detectable levels of hMPV specific antibodies, and yet hMPV infections in adults can lead to severe LRTI requiring medical attention, such as those seen with ILI in this study (Chapter 3 and 6). Infants and young children also experience hMPV infections at a time when maternal antibodies should be protective, as is the case for hRSV (Chapter 3 and 6).

These data demonstrate that, as with hRSV, immunity against hMPV is likely to be incomplete and therefore not protective against reinfection (Alvarez & Tripp, 2005). hMPV may also possess several mechanisms for evading host immune responses, although what these are is not yet clear, especially since hMPV lacks the non-structural genes found in the pneumoviruses. It is highly likely, however, that the attachment glycoprotein (G) plays an important role in evasion from antibody recognition as discussed later.

Despite the apparent ability of hMPV to cause reinfections in humans, a number of studies using experimentally infected animals have shown that protection against challenge with homologous virus can be achieved, with partial protection against heterologous virus strains. This may represent differences between experimental and natural infection and also between natural host and laboratory animals.

The nature of the antibodies raised during infection in terms of their affinity, avidity, neutralising capabilities and longevity should be investigated. Neutralising antibodies can be easily measured throughout the course of infection and beyond through the use of a robust virus neutralisation assay. Affinity maturation and antibody avidity against hMPV or its individual proteins could be assessed through the use of displacement ELISAs performed in the presence of increasing concentrations sodium thiocyanate (NaSCN) which disrupts antigen-antibody interactions.

However, it is not just antibody responses that should be considered, as discussed in chapter 1 of this thesis. Innate and cellular immune responses also play an important role in clearing viral infections. Alvarez *et al* has recently demonstrated that hMPV can persist in the respiratory tracts of experimentally infected mice for up to 60 days post infection despite the presence of neutralising antibodies. Persistence was associated with the delayed onset of CTL responses. Depletion of T cells or NK cells also resulted in increased levels of virus replication(Alvarez et al., 2004, Alvarez & Tripp, 2005). Furthermore a number of studies have shown that hMPV is a poor inducer of inflammatory cytokines, compared to hRSV in both animals and humans (Domachowske et al., 2002, Guerrero-Plata et al., 2005, Laham et al., 2004). Whether these apparently dampened cellular responses contribute to the ability of hMPV to cause reinfections in not yet known, and at present a thorough investigation of innate and T cell responses to hMPV is still to be conducted.

Analysis of T cell function can be performed in a number of ways. T cell proliferation assays can be used to measure the clonal expansion of T cells in response to antigen stimulation, and the cytolytic effects of T cells measured through the use of a chromium release assay, which measures the ability of T cells to lyse chromium labeled antigen expressing cells. ELIspot assays can also be used to measure the release of specific cytokines.

To fully evaluate several aspects of hMPV reinfection however, including, severity, frequency, and duration of illness, immunity, lineage, and strain type, studies on cohorts or individuals conducted over a number of years is required.

8.4. Variability of G

HMPV G has a very high degree of nucleotide and amino acid variation (28-37% amino acid homology between A and B lineages) (Chapter 4).This is greater than that observed for hRSV, which is reported to be 38% between hRSV A and B subtypes). As with hRSV G the majority of mutations occur in the extra cellular domain of the protein with nucleotide insertions or deletions resulting in frame shift mutations which often drastically alter the appearance of the protein and may result in truncation. The extra cellular domain also contains an unusually high degree of O-linked glycosylation which may mask potentially immunodominant epitopes, the heterogeneity of this glycosylation may also contribute further to the variability of this protein.

Given the short time period over which hMPV G gene sequence has been obtained thus far it is not possible to determine whether the variation observed in G is due to a selective pressure on the virus, such as immune pressure, or whether it represents a pool of viruses which, although they differ in this gene, are genetically stable. Evidence supporting either hypothesis can only be generated from accumulating temporal data and the comparison of synonymous/ non-synonymous ratios as in chapter 4. In vitro analysis can also be conducted. A number of studies have used polyclonal and monoclonal antibodies in hRSV cell culture systems and shown that amino acid mutations frequently arise and are often associated with developing resistance to neutralisation (Rueda et al., 1991). Such techniques could be employed to investigate the effects of immunological pressure in the hMPV G protein.

Phylogenetic analysis of G was used in this work to address the question of strain diversity, and has shown that whilst a large degree of variation exists within this protein, strains from different populations, countries and years are genetically very similar. Strains circulating within the UK are therefore genetically similar to strains circulating elsewhere in the world, as are strains circulating in the general community and hospitalised children.

During any given year both A and B lineages co-circulate in the community. In one study, however, the predominant lineage switched from A to B over 2 consecutive years, but the severity of illness did not change (Agapov et al., 2006). While the sequencing strategy used in this study identified viruses belonging predominantly to the A lineage, given the number of identified cases in which G sequence could not be obtained it is not possible to

ascertain, nor has it been established elsewhere, whether one lineage is predominant over the other, or whether one is associated with a more severe clinical picture. Whether infection with either subtype of hRSV is associated with a particular clinical outcome is still under debate and a number of contradictory findings have been published (McIntosh et al., 1993, Papadopoulos et al., 2004, Walsh et al., 1997)

It is clear that the sequencing strategy used in this study requires optimisation in order to increase the success rate of obtaining full length G sequence. However, the poor sample quality is likely to be a limiting factor in this work. Due to the difficulties of obtaining full length G sequence, in future work, fragment analysis of the hyper variable region of G could be used to generate phylogenetic data. Simple sub-typing of hMPV could be achieved by sequencing a more conserved gene, or better still by a primary diagnostic able to differentiate between subtypes.

Another important feature of the hMPV G protein is the high frequency in which premature stop codons are observed in the carboxyl terminal of the protein suggesting this region of the protein is not important structurally. Furthermore the ability of hMPV to tolerate such dramatic mutations in this protein calls in to question the function of this protein and its role in receptor binding. It has been demonstrated by Biacchesi *et al* that full length clones in which the entire G gene is deleted are capable or replication in vitro, and in the URT of hamster models to levels comparable to that of wild type virus. Replication in the LRT however was somewhat inhibited (Biacchesi et al., 2005, Biacchesi et al., 2004). This suggests hMPV has alternative mechanisms for attaching to the host cell, most likely via the fusion protein.

RSV strains also often contain premature stop codons in their attachment glycoprotein's, which supports the findings of this study and suggest the data is technically sound. In a study by Rueda et al (1991) several hRSV escape mutants arose as a result of immunological pressure following several rounds of growth in the presence of a number of different monoclonal antibodies specific for the hRSV G protein. Sequence analysis revealed that 5 out of the 7 escape mutants had nucleotide changes yielding premature stop codons which resulted in truncations of 11 to 42 amino acids in the carboxyl terminal of the protein (Rueda et al., 1991). Variation in the length of the attachment proteins may therefore be a useful strategy for evasion of antibody recognition, and it would be interesting to perform similar experiments with hMPV.

8.5. Sero-reactivity of hMPV G

Due to the potential involvement of G in immune evasion, attempts were made in this project to develop the tools for analysis of human antibody reactivity against this protein looking at the potential for sero-cross reactivity between the G proteins obtained from different hMPV isolates, and mapping the immunodominant regions (Chapter 7).

A recombinant baculovirus expressing the full length G protein of hMPV was generated. The success achieved with the nucleocapsid protein however was not repeated. A high MOI of recombinant virus was required in order achieve detectable levels of protein expression, and the protein proved difficult to extract from the cellular membrane material. Attempts to optimise the level of protein expression were not very effective and the reasons for this low level of expression were not investigated further within the scope of this work. Instead a recombinant baculovirus expressing a truncated extra cellular domain of G fused to the Fc fragment of human IgG was generated. This protein was expressed in very high levels in both glycosylated and un-glycosylated forms.

Reactivity of a panel of human sera with known antibody status for the hMPV N protein was analysed against both the hMPV G-FL and G(s)-Fc. Only 2 human sera were consistently reactive against the G-FL protein by ELISA, IF and western blot analysis. However no human sera were reactive against the hMPV G(s)-Fc protein suggesting the integrity of the protein was not maintained when fused to the IgG Fc fusion protein. Alternatively antibody reactivity may be targeted towards the membrane and cytoplasmic regions of the protein. This data indicates that hMPV G(s)-Fc is therefore unsuitable for use in immunoassays. In addition the polyclonal rabbit serum raised against this protein is also unlikely to be a useful diagnostic or research reagent.

The lack of antibody reactivity by a majority of the human serum tested against hMPV G-FL may not be surprising given the extent of amino acid variation observed (Chapter 4). Furthermore western blot analysis revealed that the human serum only reacted with the un-glycosylated species of hMPV G-FL. the rabbit α-hMPV total antiserum however appeared to recognise both, although the glycosylated species was less well recognised than the un-glycosylated. The reasons for the difference between human and rabbit serum

is not known but this data is somewhat consistent with that published by Bastian *et al* (2004) where polyclonal guinea pig antiserum raised against hMPV infected cells failed to react with the highly glycosylated species of recombinant baculovirus expressed G protein by Immuno-precipitation (Bastien et al., 2004).

These observations suggest that antibodies to the glycosylated forms of G may be poorly induced, and that glycosylation may interfere with antibody recognition of the G protein. As demonstrated in chapter 4 a large degree of heterogeneity exists in the glycosylation profiles of G proteins from different strains of hMPV. This heterogeneity may further contribute to the lack of antibody recognition. However, it should also be kept in mind that differences between human and insect cell glycosylation may also contribute to these results and this data should be treated with caution.

Beyond antibody recognition of hMPV G-FL by the rabbit α-hMPV total antiserum, and human antiserum, and its association with cellular membranes, we did not evaluate whether the integrity of G-FL was maintained. There are however numerous examples in the literature of other viral glycoproteins such as hRSV G and the influenza haemagglutinin being successfully expressed in the recombinant baculovirus system and used for the development of immunoassays, protein characterisation and vaccination (Buraphacheep et al., 1997, Crawford et al., 1999, Sugiura et al., 2001). Work on the influenza heaemagglutinin in particular has demonstrated that the baculovirus system is capable of expressing biologically functional glycoproteins. The integrity of hMPV G could have been assessed through the analysis of the extent and nature of its glycosylation, its cellular localization and whether oligomerisation of this protein occurs.

Due to the differences in glycosylation observed between insect and mammalian cells (Sullender & Britt, 1996).The expression of hMPV G-FL in a mammalian cell expression system was also investigated using a plasmid based expression system (Chapter 7). Although this was only preliminary work analysis by immunofluorescence revealed the protein was successfully expressed and it paves the way for the production of constitutively expressing cell lines which can be used for immunoassays, and analysis of protein trafficking and localisation within the mammalian cell. Recombinant vaccinia viruses have also been previously used for the expression of hRSV G (Feldman et al.,

2001, Murphy et al., 1988, Wertz et al., 1989), and may prove useful when larger quantities of mammalian expressed G protein are required.

Another approach being undertaken is the construction of pseudo-retrovirus particles expressing the hMPV G and F proteins. This is being done in collaboration with Dr Nigel Temperton (Wohl Virion Centre, UCL) and is currently only in its early stages. It is hoped these constructs will offer the opportunity to develop assays for measuring neutralising antibodies targeted against the G and F proteins, as well as providing a means of investigating susceptible cell lines and putative receptors.

8.6. Vaccine development

At this point it is still debatable as to whether hMPV vaccine development is worthwhile. While hMPV is clearly an important pathogen, the most at risk groups of severe illness are infants or young children who are often too young for vaccination to be of any benefit. Furthermore the ability of hMPV to cause repeated infections in older children and adults, and infect infants at a time when maternal antibodies should be protective raises the question as to whether protective immunity can be achieved through vaccination when it is clearly not achieved through natural infection. Repeat infections with hRSV, however, generally follow a milder cause of illness (Glezen et al., 1986) , vaccination may therefore serve to reduce the severity of disease rather than prevent it altogether.

Furthermore, given that a suitable vaccine candidate for hRSV (a far more important and well characterised virus), is yet to be found, it is likely, due to the many similarities of these two viruses that hMPV vaccine development will be equally problematic, and is further hampered by the limited availability of data on the pathogenesis of hMPV in its natural host. A number of groups have already embarked upon the search for a suitable vaccine candidate for hMPV through the use of reverse genetics and the construction of chimeric viruses which may allow for the more rapid identification and production of a number of monovalent or perhaps more useful polyvalent vaccine candidates (Biacchesi et al., 2005, Biacchesi et al., 2004, MacPhail et al., 2004, Tang et al., 2005).

8.7. Summary

The work presented in this thesis contributes to the overall understanding of hMPV, and is the largest study of its type conducted within the UK. This work demonstrates the importance of hMPV as a pathogen through the development and application of a number of research tools and diagnostics and investigation of viral protein expression in recombinant systems. Sequence analysis of the hMPV G highlights a number of potentially important characteristics of this protein which should be considered for vaccine development. Moreover this study highlights a number of key areas which could be the focus of future research.

Appendix 1

Ethical Approval

The respiratory and serum samples analysed in this work where from patients who were not recruited specifically for this project. The studies used in this thesis were set up long before the commencement of this PhD , and there was no involvement in the actual patient sampling or clinical study design. The work for this PhD project fully complies with all existing UK legislation and Health Protection Agency policies

The Royal College of General Practitioners (RCGP) Clinical Surveillance of influenza like illness(ILI) : This is a surveillance scheme that has been running in England since 1993 and has pioneered linked clinical-virological investigation for the presence of respiratory viruses (Influenza, hRSV, hMPV and others) in the community. Swabs and serum are taken as part of these schemes and the types of investigations are documented in several publications (Ellis et al., 1997, Stockton et al., 1998, Stockton et al., 2002, Zambon et al., 2001).

As these samples are taken as part of normal diagnostic work up, ethical committee submission is not required for investigation of respiratory viruses.

Leicester Paediatric study: This is a study of infant hospitalisation related to Influenza, hRSV , hMPV and other respiratory viral pathogens documented in several publications (Nicholson et al., 2006, Nicholson et al., 2003). As these samples are taken as part of normal diagnostic work up, ethical committee submission is not required for investigation of respiratory viruses.

Royal Free Immunocompromised study: These samples where taken as part of normal diagnostic work up for the investigation of respiratory illness, ethical committee submission is therefore not required for investigation of respiratory viruses.

Age Stratified Serum: These serum were submitted to the HPA for testing for MMR, Parvovirus B19 or rash virus diagnosis and not necessarily for respiratory virus infection. The sera used however were residual and not destined for any other testing, due only for discard, data was provided anonymously with the only detail being the age of the patient at the time of sampling. The latest ethics guidance document from The Royal College of

Pathologists "*Interim guidance on the use of clinical samples retained in the pathology laboratory*" issued in Nov 2004 specifically identifies testing of residual material for validation of new tests as desirable and is exempt from requirements for specific ethical approval.

Data Security

The Health Protection Agency is fully compliant with Caldicott guidelines, which were laid out in the 1997 December report (Department of Health & Caldicott, 1997). The HPA is unreservedly committed to preserving medical confidentiality in all aspects of its work, both in direct contact with clinicians and patients, and in aggregating data for surveillance and research purposes.

Appendix 2

hMPV G gene sequences used in the phylogenetic analysis data presented in chapter 4 of this thesis. Sequences were down loaded from http://www.ncbi.nlm.nih.gov/

Strain	Accession no	Reference
NL001(A1)*	AF371337	(van den Hoogen et al., 2001)
NL1700(A2)*	AY296021	(van den Hoogen et al., 2004)
Nl199(B1)*	AY296034	
NL194(B2)*	AY296040	
NL196	AY296042	
NL202	AY296020	
NL181	AY296022	
NL182	AY296041	
NL193	AY296023	
NL195	AY296026	
NL900	AY296044	
NL901	AY296038	
NL1001	AY296019	
NL1100	AY296035	
NL1200	AY296036	
NL2101	AY296039	
NL2201	AY296029	
NL2301	AY296031	
NL2401	AY296030	
NL2901	AY296032	
NL293	AY296024	
NL296	AY296027	
NL301	AY296045	
NL302	AY296033	
NL393	AY296025	
NL396	AY296028	
NL401	AY296046	
NL501	AY296037	
NL697	AY296043	
UK5O1	AY296047	
BR201	AY296014	

FL301	AY296016	
FL401	AY296015	
FL801	AY296017	
FL1001	AY296018	
CAN3402	AY574225	(Bastien et al., 2004)
CAN3402	AY574225	
CAN4002	AY574226	
CAN5802	AY574227	
CAN9702	AY574228	
CAN9783	AY574249	
CAN9875	AY297748	
CAN9980	AY574247	
CAN9981	AY574224	
CAN16402	AY574229	
CAN18202	AY574230	
CAN18702	AY574231	
CAN18802	AY574232	
CAN19402	AY574253	
CAN19702	AY574234	
CAN20802	AY574235	
CAN21502	AY574236	
CAN21602	AY574237	
CAN21902	AY574238	
CAN 22702	AY 574239	
CAN 34702	AY 574240	
CAN 34802	AY 574241	
CAN 43202	AY 574242	
CAN 46402	AY 574243	
CAN 53202	AY 574244	
CAN 54702	AY 574246	
CAN 53402	AY 574245	
HMPV8099	AY 485250	(Peret et al., 2004)
HMPV 8397	AY 485223	
HMPV13200	AY 485232	

HMPV 16200	AY 485033	
HMPV 17200	AY 485234	
HMPV 222001	AY 485236	
HMPV 232001	AY 485238	
HMPV 282001	AY 485239	
HMPV 292001	AY 485240	
HMPV 312001	AY 485241	
HMPV 332001	AY 485242	
HMPV 731998	AY 485243	
HMPV 741998	AY 485244	
HMPV 751998	AY 485245	
HMPV 761998	AY 485246	
HMPV 771998	AY 485247	
HMPV 781998	AY 485248	
HMPV 791998	AY 485249	
HMPV 811999	AY 485251	
HMPV 821997	AY 485252	
HMPV 1932002	AY 485235	
HMPV 2282002	AY 485237	
HMPV 8631602	AY 485254	
HMPV 8844802	AY 485255	
HMPV 8847002	AY 485256	
JPSO3480	AY 530092	(Ishiguro et al., 2004)
JPSO 3191	AY 530094	
JPSO 3240	AY 530095	
JPSO 276	AY 530089	
JPSO 3176	AY 530090	
JPSO 3178	AY 530091	
Q01419	AY 327803	(Mackay et al., 2003)
Q01629	AY 327810	
Q01634	AY 327806	
Q01641	AY 327804	
Q01718	AY 327802	
Q01726	AY 327805	

Q01729	AY 327809
Q01799	AY 327807
Q01225	AY 327808

*Reference strains

Amino acid alignment of hMPV G protein sequences obtained from the Community Study

```
Majority    MEVKVENIRAIDMLKARVKNRVARSKCFKNASLILIGITTLSIALNIYLIINYTMQKNTSESEHHTSSSP
                      10        20        30        40        50        60        70
JMRCGP558   ...........................................................I..T.........P.
JMRCGP1400  ...........................................................I..T.........P.
NL1700GA2   ...........................................................I..T.........P.
JMRCGP9935  ..............................................................E..........
JMRCGP336   .........T...................................................E..........
JMRCGP2184  .........T...................................................E..........
JMRCGP4661  .........T...................................................E..........
JMRCGP559   .........T.....................T.............................G..........
NL100BA1    .........T.........................V.....................K..............
NL199GB1    ...R.........F..KI...IRS.R.YR..T.....L.A..M....F...DHATLR.MIKT.NCANMPS
RCGP348034  ...R.........F..KI...IRS.R.YR..T.....L.A..M....F...DHATSR.MIKT.NCANMPS
NL194GB2    ...R.........F..KM...IRS...YR..T.....L.A..M....F...D.A.L..MTKV..CVNMP.

Majority    MESSKETPTVPTDNSDTNPGSQYPTQQSTEGSTLHFAASASSPETEPTSTPDTTSRPPFVDTHTTPPSAS
                      80        90       100       110       120       130       140
JMRCGP558   T..N...S.I.I..P.I..N..H.......SL..YP.S.V.PS....A...GI.N.LSS..RS..Q..G.
JMRCGP1400  T..N...S.I.I..P.I..N..H.......SL..YP.S.V.PS....A...GI.N.LS...RS..Q..E.
NL1700GA2   T.PN..AS.IS...P.I..S..H.......NP..NP.....PS....A......N.LSS..RS.AQ..E.
JMRCGP9935  ....R......M.....................................................G..
JMRCGP336   ....R......M.....................P.....................L..........
JMRCGP2184  ....R......I.....................S.....................L..........
JMRCGP4661  ....R......M.....................................S..................
JMRCGP559   ....R....I.M..............P......Y.....................................
NL100BA1    ....R............SSP.H..........Y.....................N..............
NL199GB1    A.P..K..MTS.AGPN.K.NP.QA..WT..N..SPV.TPEGH.Y.GT.Q.S...APQQTT.K..A.LKST
RCGP348034  A.P..KA.MTS.AGPS.K.NP.QA..WT..N..SPA.TPEGH.Y.GT.Q.....APQQTT.KY.AL.KST
NL194GB2    V.P..K..MTSAVDLN.K.NP.QA..LAA.D..SLA.T.EDHLH.GT.P...A.VSQQTT.EY..LLRST

Majority    RTRTSPAVHTKNNPRISSRTHSPPWATTRTVRGTTTLRTSSTRKRPSTASVQPDSSATTHKHEEASPASP
                     150       160       170       180       190       200       210
JMRCGP558   ..KIN.T..K..ISSTV...Q..TRT.AKAATRA.A..M...GE..T.T...S...T..QN...TGS.N.
JMRCGP1400  ..K.NST..K..ISSTI...Q...RT.AKA.PR..AF.....GE..T.T...S...T.AQN...TGS.N.
NL1700GA2   ..K.K.T...I...NTA.S.Q...RT..KAI.RA..F.M...G...T.TL..S...T..QN...TGS.N.
JMRCGP9935  ............L...P........M..............I.........................V..
JMRCGP336   .K..............P........M..............I.........................V..
JMRCGP2184  ................P.A.....................I.........................V..
JMRCGP4661  ................P.....................................................V..
JMRCGP559   ............K..L.......VM..M.....................................
NL100BA1    ..K............T........R.....A.R.....................I......N.........
NL199GB1    NEQITQTTTE.KTI.ATTQKREKGKEN.NQTTS.AATQ.TN.TNQIRN..ETITT.DRPRTDTTTQSSEQ
RCGP348034  NEQITQTTTE.KTT.ATTQKREKGKEN.NQTTS.AATQ.TN.TNQIRN.RRDNHNIRQ.QN
NL194GB2    NRQ.TQTTTE.KPTGATTKK-----E..TRTTS.AATQ.LN.TNQT.YVREATTT..RSRNSATTQSSDQ

Majority    QTSASTARPQRKGMEASTSTTYNQTS-----
                      220       230       240
JMRCGP558   .A....MQK
JMRCGP1400  .A....M
NL1700GA2   .A....MQN
JMRCGP9935  .....I.....................
JMRCGP336   ...........................
JMRCGP2184  .A.........................
JMRCGP4661  ..........G................
JMRCGP559   ....................LQFKLS
NL100BA1    ......T.I...SV..N.........
NL199GB1    T.R.TDPSSPPHHA
RCGP348034
NL194GB2    T.Q.ADPSS.PHHTQK..T....TDTSSPSS
```

Amino acid alignment of hMPV G protein sequences obtained from the hospitalised Children's study

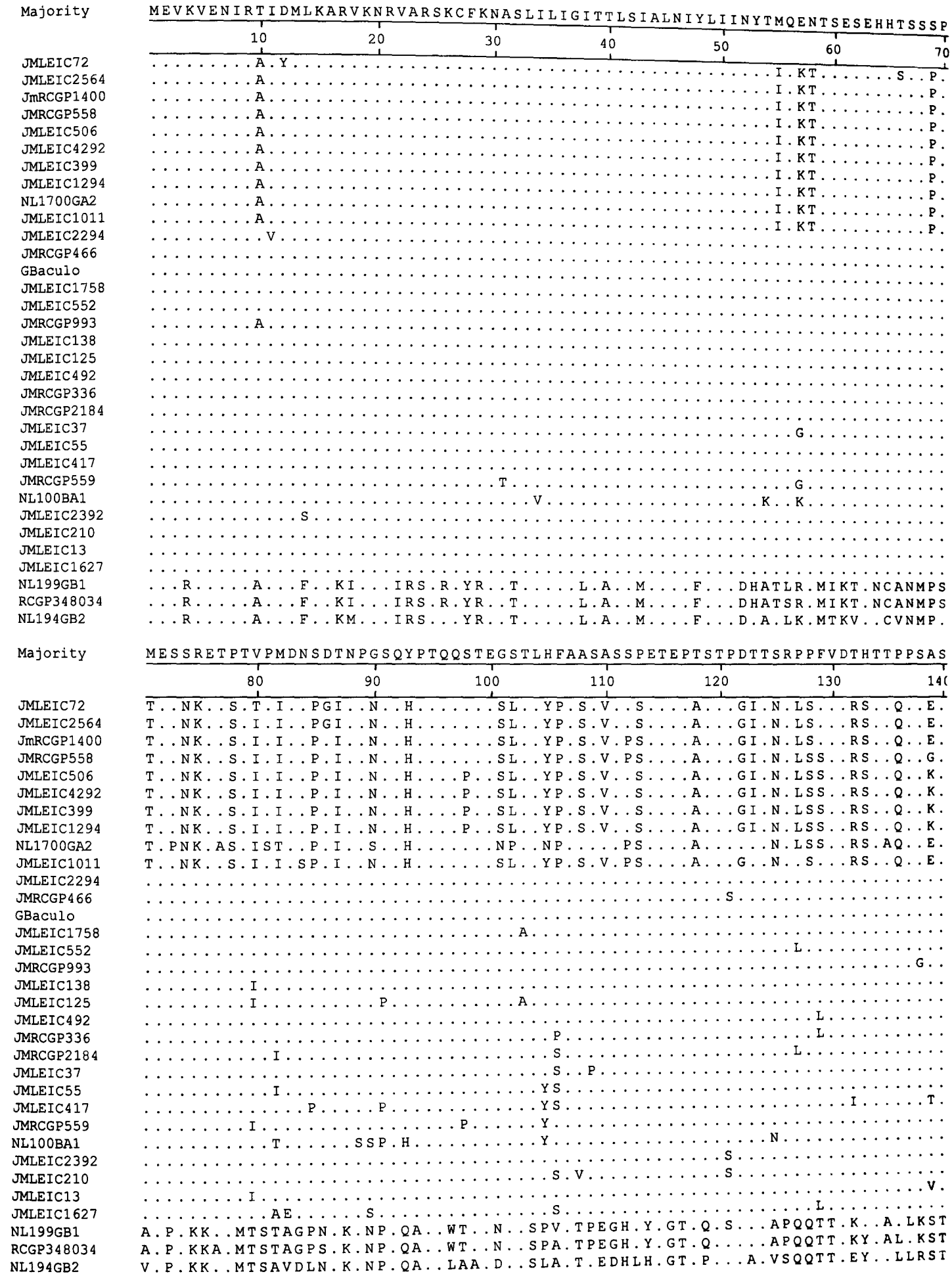
```
Majority   MEVKVENIRTIDMLKARVKNRVARSKCFKNASLILIGITTLSIALNIYLIINYTMQENTSESEHHTSSSP
                    10        20        30        40        50        60        70
JMLEIC72   .........A.Y.....................................................I.KT.......S..P.
JMLEIC2564 .........A.....................................................I.KT.........P.
JMLEIC506  .........A.....................................................I.KT.........P.
JMLEIC4292 .........A.....................................................I.KT.........P.
JMLEIC399  .........A.....................................................I.KT.........P.
JMLEIC1294 .........A.....................................................I.KT.........P.
NL1700GA2 .........A.....................................................I.KT.........P.
JMLEIC1011 .........A.....................................................I.KT.........P.
JMLEIC1758 ..................................................................................
GBaculo    ..................................................................................
JMLEIC552  ..................................................................................
JMLEIC138  ..................................................................................
JMLEIC125  ..................................................................................
JMLEIC37   ..............................................................G...................
JMLEIC492  ..................................................................................
JMLEIC2294 ..........V.....................................................................
JMLEIC55   ..................................................................................
JMLEIC417  ..................................................................................
NL100BA1  ..................................V.....................K..K.....................
JMLEIC2392 ............S.....................................................................
JMLEIC210  ..................................................................................
JMLEIC13   ..................................................................................
JMLEIC1627 ..................................................................................
NL199GB1  ...R.....A...F..KI...IRS.R.YR..T.....L.A..M....F...DHATLR.MIKT.NCANMPS
NL194GB2  ...R.....A...F..KM...IRS...YR..T.....L.A..M....F...D.A.LK.MTKV..CVNMP.

Majority   MESSRETPTVPMDNSDTNPGSQYPTQQSTEGSTLHFAASASSPETEPTSTPDTTSRPPFVDTHTTPPSAS
                    80        90        100       110       120       130       140
JMLEIC72   T..NK..S.T.I..PGI..N..H.......SL..YP.S.V..S....A...GI.N.LS...RS..Q..E.
JMLEIC2564 T..NK..S.I.I..PGI..N..H.......SL..YP.S.V..S....A...GI.N.LS...RS..Q..E.
JMLEIC506  T..NK..S.I.I..P.I..N..H....P..SL..YP.S.V..S....A...GI.N.LSS..RS..Q..K.
JMLEIC4292 T..NK..S.I.I..P.I..N..H....P..SL..YP.S.V..S....A...GI.N.LSS..RS..Q..K.
JMLEIC399  T..NK..S.I.I..P.I..N..H....P..SL..YP.S.V..S....A...GI.N.LSS..RS..Q..K.
JMLEIC1294 T..NK..S.I.I..P.I..N..H....P..SL..YP.S.V..S....A...GI.N.LSS..RS..Q..K.
NL1700GA2  T.PNK.AS.IST..P.I..S..H.......NP..NP.....PS....A......N.LSS..RS.AQ..E.
JMLEIC1011 T..NK..S.I.I.SP.I..N..H.......SL..YP.S.V.PS....A...G..N..S...RS..Q..E.
JMLEIC1758 ..............................A...................................................
GBaculo    ..................................................................................
JMLEIC552  ..................................................................L...............
JMLEIC138  .........I........................................................................
JMLEIC125  .........I..........P...........A..................................................
JMLEIC37   ..................................S..P..............................................
JMLEIC492  ..................................................................L...............
JMLEIC2294 ..................................................................................
JMLEIC55   ...........I......................YS..................................................
JMLEIC417  ..............P.....P............YS.........................I......T.
NL100BA1   ...........T......SSP.H..........Y.....................N.....................
JMLEIC2392 ..................................................S...............................
JMLEIC210  ..................................S.V............S...............................
JMLEIC13   .........I........................................................................V.
JMLEIC1627 ...........AE......S.............S.....................L.....................
NL199GB1   A.P.KK..MTSTAGPN.K.NP.QA..WT..N..SPV.TPEGH.Y.GT.Q.S...APQQTT.K..A.LKST
NL194GB2   V.P.KK..MTSAVDLN.K.NP.QA..LAA.D..SLA.T.EDHLH.GT.P...A.VSQQTT.EY..LLRST
```

```
Majority      RTKTSPAVHTKNNPRTSPRTHSPPRATTRTVRGTTTLRTSSTRKRPSTASVQPDSSATTHKHEEASPVSP
                                150       160       170       180       190       200       21
JMLEIC72      ....NRT..K..ISS.VS..Q....T.AKV.PRA.AP.....GE..T.TP......T..QN...TGSAN.
JMLEIC2564    ....NRT..K..ISS.VS..Q....T.AKV.PRA.A......GE..T.TP......T..QN...TGSAN.
JMLEIC506     ....T.T..KR.I.S.VS..Q....T.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN.
JMLEIC4292    ....T.T..KR.I.S.VS..Q....T.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN.
JMLEIC399     ....T.T..KR.I.S.VS..Q....T.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN.
JMLEIC1294    ....T.T..KR.I.S.VS..Q....T.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN.
NL1700GA2     ....K.T...I...N.ASS.Q....T..KAI.RA..F.M...G...T.TL..S...T..QN...TGSAN.
JMLEIC1011    ....NST..K.KTSQVQFLE...HHGQQ.RRSPEPPPFARAAQEKDQPQHQSSLTT.PQP.IMKKQV
JMLEIC1758    ..R.........L.I.........M.............I.........................
GBaculo       ..R.........L.I........L.M.............I.........................
JMLEIC552     ............L.I........W.M.............I.........................
JMLEIC138     ..R.........L.I.S......W.M.............I.........................
JMLEIC125     ..R...........I.S......W.M...A.........................................
JMLEIC37      ..R.....................W.M.............I.........................
JMLEIC492     ..R...........I........W.M.....................................
JMLEIC2294    ......T........I........W.....................................M..
JMLEIC55      ..R.................F..W.........................A.....................
JMLEIC417     ..R.................L..W.M.............................T...........L
NL100BA1      ................S............A.R.....................I......N.....A..
JMLEIC2392    ..R........KQSKDK.QNTF.TMGNDKDGPWDHHSPHKQHK.KTVHSISPTRQQRNNPQTRRSK.SE.
JMLEIC210     ..R........KRSKDK.QNTF.TMGNDKDGPWDHHSPHKQHK.KTVHSISPTRQQRNNPQTRKSK.SE.
JMLEIC13      ..R........KQSKDKLQNTF.TMGNDKDGPWNHHSPHKQHK.KTVHSISPT
JMLEIC1627    .........K.TTQGQA.EHVPHHGQQQGRSTEPPLSAQAAQE.DRPQHQSN.TAAQQSTNTKKQVQ
NL199GB1      NEQITQTTTE.KTI.ATTQKREKGKEN.NQTTS.AATQ.TN.TNQIRN..ETITT.DRPRTDTTTQSSEQ
NL194GB2      NRQ.TQTTTE.KPTGATTKK-----E..TRTTS.AATQ.LN.TNQT.YVREATTT..RSRNSATTQSSDQ

Majority      QTSASTARPQRKGMEASTSTT-NQT------
                          220       230       240
JMLEIC72      .A....MQN.HTNIARPN
JMLEIC2564    .A....MQN.HTNIARPN
JMLEIC506     .A..N.M
JMLEIC4292    .A..N.M
JMLEIC399     .A..N.M
JMLEIC1294    .A..N.M
NL1700GA2     .A....MQN
JMLEIC1011
JMLEIC1758    ......................H...S
GBaculo       ......................Y...S
JMLEIC552     .....I................Y...S
JMLEIC138     ......................Y...S
JMLEIC125     ......................Y...S
JMLEIC37      ......................H...S
JMLEIC492     ......T..............Y...S
JMLEIC2294    ..........G..........Y...S
JMLEIC55      ....G..........T.....Y...S
JMLEIC417     ......................H...S
NL100BA1      ......T.I...SV..N....Y...S
JMLEIC2392    ANICKHSKTT.E.YGGQHINNIQPN
JMLEIC210     ANICKHSKTT.E.YGGQHINNIQPN
JMLEIC13
JMLEIC1627
NL199GB1      T.R.TDPSSPPHHA
NL194GB2      T.Q.ADPSS.PHHTQK..T..Y.TDTSSPSS
```

Amino acid alignment of sequences generated in this study from the community and hospitalised children's studies.



```
Majority        RTRTSPAVHTKNNPRTSPRTHSPPWATTRTVRGTTTLRTSSTRKRPSTASVQPDSSATTHKHEEASPVSP
                          150       160       170       180       190       200       21(
JMLEIC72        ..K.NRT..K..ISS.VS..Q...RT.AKV.PRA.AP.....GE..T.TP......T..QN...TGSAN.
JMLEIC2564      ..K.NRT..K..ISS.VS..Q...RT.AKV.PRA.A......GE..T.TP......T..QN...TGSAN.
JmRCGP1400      ..K.NST..K..ISS.IS..Q...RT.AKA.PR..AF.....GE..T.T...S...T.AQN...TGSAN.
JMRCGP558       ..KIN.T..K..ISS.VS..Q..TRT.AKAATRA.A..M...GE..T.T...S...T..QN...TGSAN.
JMLEIC506       ..K.T.T..KR.I.S.VS..Q...RT.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN.
JMLEIC4292      ..K.T.T..KR.I.S.VS..Q...RT.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN.
JMLEIC399       ..K.T.T..KR.I.S.VS..Q...RT.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN.
JMLEIC1294      ..K.T.T..KR.I.S.VS..Q...RT.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN.
NL1700GA2       ..K.K.T...I...N.ASS.Q...RT..KAI.RA..F.M...G...T.TL..S...T..QN...TGSAN.
JMLEIC1011      ..K.NST..K.KTSQVQFLE...HHGQQ.RRSPEPPPFARAAQEKDQPQHQSSLTT.PQP.IMKKQV
JMLEIC2294      ..K...T.......I.....................................................M..
JMRCGP466       .............I.....................................................
GBaculo         ............L.I.......L.M.............I.....................
JMLEIC1758      ............L.I.......R.M.............I.....................
JMLEIC552       ..K.........L.I.........M.............I.....................
JMRCGP993       ............L.I.........M.............I.....................
JMLEIC138       ............L.I.S.......M.............I.....................
JMLEIC125       .............I.S.......M...A.....................................
JMLEIC492       .............I.........M.....................................
JMRCGP336       .K...........I.........M.............I.....................
JMRCGP2184      .............I...A.....................I.....................
JMLEIC37        ........................M.............I.....................
JMLEIC55        ....................F.....................A.....................
JMLEIC417       ....................L....M.....................T.....L
JMRCGP559       ............KI.L.......VM..M.....................................A..
NL100BA1        ..K...........S......R.....A.R.....................I......N.....A..
JMLEIC2392      ..........KQSKDK.QNTF.TMGNDKDGPWDHHSPHKQHK.KTVHSISPTRQQRNNPQTRRSK.SE.
JMLEIC210       ..........KRSKDK.QNTF.TMGNDKDGPWDHHSPHKQHK.KTVHSISPTRQQRNNPQTRKSK.SE.
JMLEIC13        ..........KQSKDKLQNTF.TMGNDKDGPWNHHSPHKQHK.KTVHSISPT
JMLEIC1627      ..K......K.TTQGQA.EHVPHHGQQQGRSTEPPLSAQAAQE.DRPQHQSN.TAAQQSTNTKKQVQ
NL199GB1        NEQITQTTTE.KTI.ATTQKREKGKEN.NQTTS.AATQ.TN.TNQIRN..ETITT.DRPRTDTTTQSSEQ
RCGP348034      NEQITQTTTE.KTT.ATTQKREKGKEN.NQTTS.AATQ.TN.TNQIRN.RRDNHNIRQ.QN
NL194GB2        NRQ.TQTTTE.KPTGATTKK-----E..TRTTS.AATQ.LN.TNQT.YVREATTT..RSRNSATTQSSDQ

Majority        QTSASTARPQRKGMEASTSTT-NQT------
                          220       230       240
JMLEIC72        .A....MQN.HTNIARPN
JMLEIC2564      .A....MQN.HTNIARPN
JmRCGP1400      .A....M
JMRCGP558       .A....MQK
JMLEIC506       .A..N.M
JMLEIC4292      .A..N.M
JMLEIC399       .A..N.M
JMLEIC1294      .A..N.M
NL1700GA2       .A....MQN
JMLEIC1011
JMLEIC2294      ..........G..........Y...S
JMRCGP466       ..........G..........Y...S
GBaculo         ....................Y...S
JMLEIC1758      ....................H...S
JMLEIC552       .....I..............Y...S
JMRCGP993       .....I..............Y...S
JMLEIC138       ....................Y...S
JMLEIC125       ....................Y...S
JMLEIC492       ......T.............Y...S
JMRCGP336       ....................Y...S
JMRCGP2184      .A..................Y...S
JMLEIC37        ....................H...S
JMLEIC55        ....G..........T.....Y...S
JMLEIC417       ....................H...S
JMRCGP559       ....................LQFKLS
NL100BA1        ......T.I...SV..N....Y...S
JMLEIC2392      ANICKHSKTT.E.YGGQHINNIQPN
JMLEIC210       ANICKHSKTT.E.YGGQHINNIQPN
JMLEIC13
JMLEIC1627
NL199GB1        T.R.TDPSSPPHHA
RCGP348034
NL194GB2        T.Q.ADPSS.PHHTQK..T..Y.TDTSSPSS
```

Amino acid alignment of all hMPV subtype B G protein Sequences

```
Majority    MEVRVENIRAIDMFKAKMKNRIRSSKCYRNATLILIGLTALSMALNIFLIIDYATLKNMTKVEHCVNMPP
                      10        20        30        40        50        60        70
NL501       ..................I.......R.............................H...R..I.T.N.A....
NL1200      ..................I.......R.............................H...R..I.T.N.A....
NL199B1     ..................I.......R.............................H...R..I.T.N.A....
NL1100      ..................I.......R.............................H...R..I.T.N.A...S
hMPV332001  ..................I.......R.............................H...R..I.T.N.A...S
RCGP480341  ..................I.......R.............................H...R..I.T.N.A...S
NL900       ..................I.......R.............................H..SR..I.T.N.A...S
UK501       ..................I.......R.............................H..SR..I.T.N.A...S
hMPV741998  ..................I.......R.............................H..SR..I.T.N.A...S
hMPV731998  ..................I.......R.............................H..SR..I.T.N.A...S
hMPV771998  ..................I.......R.............................H..SR..I.T.N.A...S
hMPV781998  ..................I.......R.............................H..SR..I.T.N.A...S
NL401       ..................I.......R.............................H..SR..I.T.N.A...S
hMPV761998  ..................I.......R.............................H..SR..I.T.N.A...S
CAN9980     ..................I.......R.............................H..SR..I.T.N.A...S
hMPV751998  ..................I.......R.............................H..SR..I.T.N.A...S
CAN9875     ..A...............I.......R.............................H..SR..I.T.N.A...S
NL697       ..................I.......R.............................H..SR..I.T.N.A...S
NL194B2     ..................I.......R.............................H..SR..I.T.N.A...S
NL182       .........T........I.......R.............................H..SR..I.T.N.A...S

Majority    VEPSKKTPMTSAVDPNTKPNPQQATQLTTEDSTSLAATLEDHLHTGTTPTPDATVSQQTTDEHTTLLRST
                      80        90        100       110       120       130       140
NL501       A...R......TAG.............W...N...P...P.G.......Q...T.AP.....K..A.PK..
NL1200      A...........TAG.............W...N..FP...S.G.......Q...T.AP.....K..A.PK..
NL199B1     A...........TAG.............W...N...PV..P.G.PY....Q.S.T.AP.....K..AP.K..
NL1100      A...........TAG.S.E.........W...N...P.....S.PY....Q...I.AP.....K..A.PK..
hMPV332001  A...........TAG.S...........W...N...P.....G.PY.E..Q...T.AP.....KY.A.SK..
RCGP480341  A.....A....TAG.S...........W...N...P...P.G.PY....Q...T.AP.....KY.A.PK..
NL900       ...............S.....................................P.....................
UK501       ...............L.....................................P.....................
hMPV741998  ...............L.....................................P.....................
hMPV731998  ...............L.....................................P.....................
hMPV771998  ...............L.....................................P.....................
hMPV781998  ...............S.....................P.....................F.............
NL401       ...............L.....................P.....G.....................P.....
hMPV761998  ..........................................P.....................P.....PI
CAN9980     ..........................................P.....................P.....PI
hMPV751998  ..........................................P.....................P.....PI
CAN9875     .............A..........................P.....................P.....PI
NL697       ...............L...L.....................S.G.P.....................P.....
NL194B2     ...............L.............AA.........S.....................Y.....P.....
NL182       ...........T..SS.G.....T..W...........S...........L........P.K...P....

Majority    NRQTTQTTAEKKPTRATTKKETTTR-----TTSTAATQTLNTTNQTSNGREATTTSARSRNNATTQSSDQ
                      150       160       170       180       190       200       210
NL501       .E.I..A.T...T..E..QRREKGKENTNQ.........T.....IR.AS.TI...D.P.TDS.....E.
NL1200      .E.I....T...T.....QRREKGKENTNQ.........T.....IR.AS.TI...D.P.TDS.....E.
NL199B1     .E.I....T...TI....Q.REKGKENTNQ.........T.....IR.AS.TI...D.P.TDT.....E.
NL1100      .E.I....T...T.....Q.REKEKENTNQ.........T......R.AS.TI...D.P.IDT.......
hMPV332001  .E.I....T...T....PQRRKKGKENTNQ.........T.....IR.AS.TI...D.P.IDI.....E.
RCGP480341  .E.I....T...T.....Q.REKGKENTNQ.........T.....IR.A.RDNHNIRQTQ.
NL900       ..........................-----.....................................
UK501       ..........................-----.....................................
hMPV741998  ..........................-----.....................................
hMPV731998  ..........................-----.....................................
hMPV771998  ..........................-----.....................................
hMPV781998  ..........................-----.....................................S.....
NL401       ......................I...-----.....................................
hMPV761998  ..........................-----.....................................
CAN9980     ..........................-----.....................................
hMPV751998  ..........................-----.....................................
CAN9875     ..........................-----.....................................
NL697       .......AT.....G..........-----.........P.....................G....N...
NL194B2     .........T.....G..........-----.....................YV.....S.........
NL182       .G......T.......IA.....NQ-----.........F......R....T.I.......D......E.
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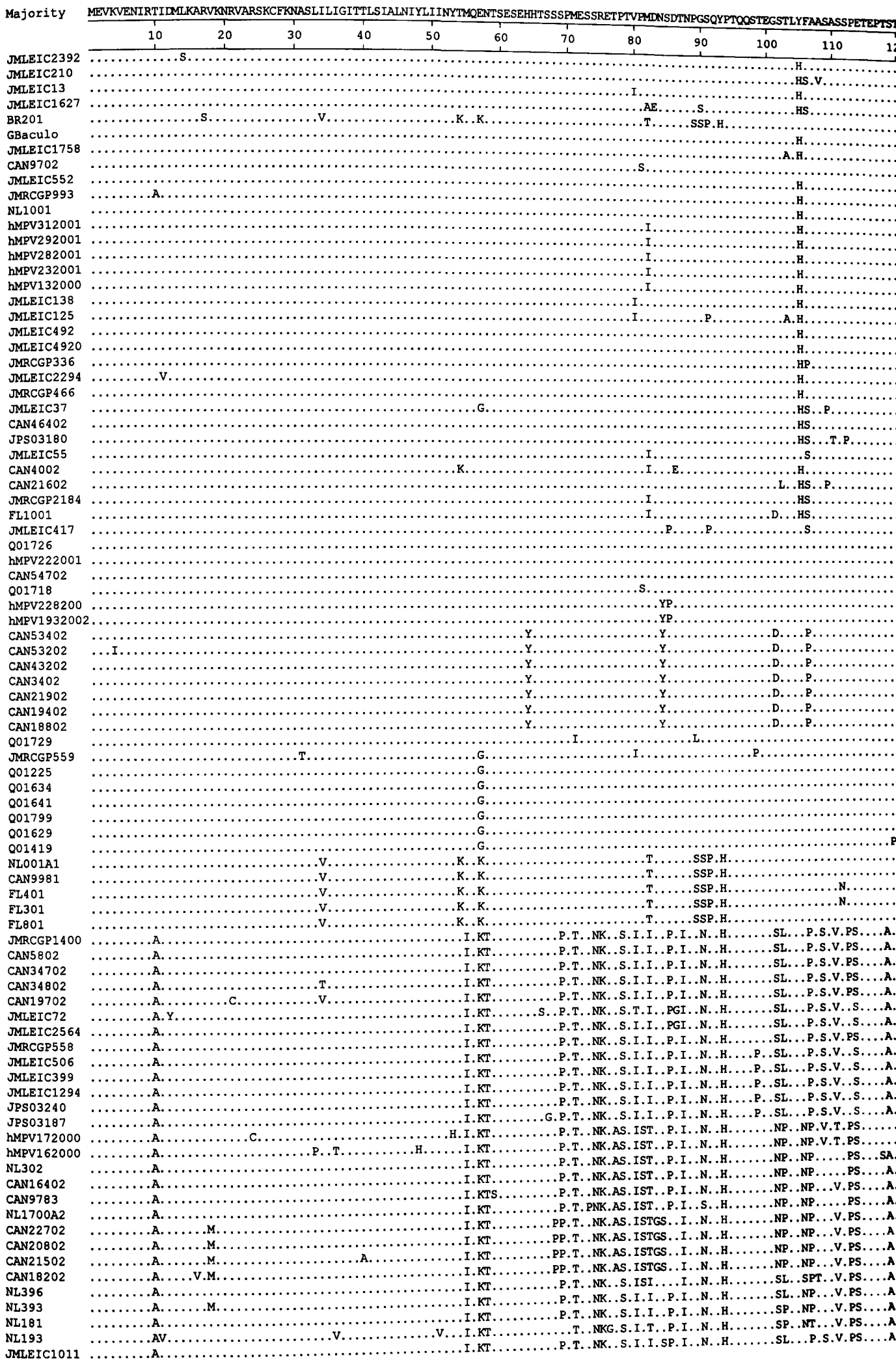
Majority      TTQAADPSSQSQHTQKSTTTTYNTDTSSPSS
                        220       230       240
NL501         ....T....PAH.A.G.AKPK
NL1200        ..R.T....PPH.A.G.AKPK
NL199B1       ..R.T....PPH.A
NL1100        ..R.T....PPH.A.SGAKPK
hMPV332001    ..R.TE.GFPPH.ARRGAGPR
RCGP480341
NL900         .....E.....................L..
UK501         .....E.N...................L..
hMPV741998    ......................I..F....
hMPV731998    ......................I.......
hMPV771998    ............................
hMPV781998    ............................
NL401         .............K..............
hMPV761998    ....................H.........
CAN9980       ....................H.........
hMPV751998    ....................H.........
CAN9875       ....................H.........
NL697         I.....S...PH........A......F...
NL194B2       ..........PH................
NL182         .N.TT.....PH.A
```

Amino acid alignment of all hMPV subtype A G protein Sequences



```
Majority    PDTTSRPPFVDTHTTPPSAsRTRTSPAVHTKNNPRISSRTHSPPWATTRTVRGTTTLRTSSTRKRPSTASVQPDSSATTHKHEEASPVSPQTSASTARPQRKGMEASTSTT-NQTS-
                      130       140       150       160       170       180       190       200       210       220       230
JMLEIC2392  S.........................KQSKDKPQNTF.TMGNDKDGPWDHHSPHKQHK.KTVHSISPTRQQRNNPQTRRSK.SE.ANICKHSKTT.E.YGGQHINNIQPN
JMLEIC210   S.........................KRSKDKPQNTF.TMGNDKDGPWDHHSPHKQHK.KTVHSISPTRQQRNNPQTRKSK.SE.ANICKHSKTT.E.YGGQHINNIQPN
JMLEIC13    ................V.........KQSKDKLQNTF.TMGNDKDGPWNHHSPHKQHK.KTVHSISPT
JMLEIC1627  ........L.........K......K.TTQGQAPEHVPHHGQQQGRSTEPPLSAQAAQE.DRPQHQSN.TAAQQSTNTKKQVQ
BR201       ....N.............K.........T........R.....A.RNHHSPHKQHK.ETVHSISPT
GBaculo     ............................L...P......L.M.............I.....................................................Y....
JMLEIC1758  ............................L...P......R.M.............I.....................................................H....
CAN9702     ............................L...P.......M.............I..G..................................................H....
JMLEIC552   ......L.............K.....L...P.......M.............I.....................................I.............Y....
JMRCGP993   ..............G.............L...P.......M.............I.....................................I.............Y....
NL1001      ..............S.....K.........L...P.......M.............I.....................................A.............Y....
hMPV312001  ............................LK..P.I.....M.....................T.....................................Y....
hMPV292001  ............................LK..P.I.....M.....................T.....................................Y....
hMPV282001  ............................LK..P.I.....M.....................T.....................................Y....
hMPV232001  ............................LK..P.I.....M.....................T.....................................Y....
hMPV132000  ............................LK..P.......M.............I.....T.....................................H....
JMLEIC138   ............................L...........M.............I.....................................................Y....
JMLEIC125   ..........................................M...A.....................................................Y....
JMLEIC492   ........L.....................P.......M.....................................T.....................Y....
JMLEIC4920  ........L.....................P.......M.....................................T.....................Y....
JMRCGP336   ........L...........K.........P.......M.............I.....................................................Y....
JMLEIC2294  ..................K...T.........P.....................................M.....G.....................Y....
JMRCGP466   S.....................P.....................................G.....................Y....
JMLEIC37    ............................T.P.......M.............I.....................................................H....
CAN46402    ............................L.T.P.......M.............I.....................................................Y....
JPS03180    ..................K..............TNP.......M.....................................................Y....
JMLEIC55    ..............................T.P...F.....................A.....................G.....T.....Y....
CAN4002     .....H.....................P.....................A.....................P..Y....
CAN21602    .........A....S....K.....P.....................I..A.....D.....R...T.....Y....
JMRCGP2184  ......L.....................P.A.....I.....A.....Y....
FL1001      ..............V.P.......M.....L.....T.....Y....
JMLEIC417   ...........I......T.....T.P...L....M.....T.....L.....H....
Q01726      ....................N.....T.P.....M..E.....A.....T.....
hMPV222001  ..............................T.P.....M.....Y....
CAN54702    ..............................T.P.....M.....Y....
Q01718      ..............................T.P.....R.M.....
hMPV228200  .N.....................K.....L.....M.....V.....Y....
hMPV1932002 .N.....................K.....L.....M.....V.....Y....
CAN53402    ..................................L.....Y....
CAN53202    ..................................L.....Y....
CAN43202    ..................................L.....Y....
CAN3402     ..................................L.....Y....
CAN21902    ..................................L.....Y....
CAN19402    ..................................L.....Y....
CAN18802    ..................................L.....Y....
Q01729      ....................R.....L...L.....M.....
JMRCGP559   ..............................K..L.....VM..M.....A.....LQFKLS
Q01225      ..............................K.....VM..M.....A.....
Q01634      ..............................K..L.....TM..M.....A.....
Q01641      ..............................LK..L.....M..M.....A.....
Q01799      ..............................L...L.....M..M.....V..A.....
Q01629      ..............................L...L.....M..M.....A.....
Q01419      .........A.....................L.....M.....L.....A.....L...
NL001A1     ....N.............K.....T.....R.....A.R.....I.....N.....A.....T.I...SV..N....Y....
CAN9981     ....N.............K.....T.....R.....A.R.....I.....N.....A.....T.I...SV..N....Y....
FL401       ....N.............K.....A.R.....A...I.....N.....A.....T.T...SV..N....Y....
FL301       ....D.............K.....A.R.....I.....N.....A.....T.T...SV..N....Y....
FL801       ....D.............K.....A.R.....I.....N.....A.....T.T...SV..N....Y....
JMRCGP1400  .GI.N.LS...RS..Q..E...K.NST..K..ISSTI...Q...RT.AKA.PR..AF.....GE..T.T...S...T.AQN...TGSAN..A....M
CAN5802     .GI.N.LS...RS..Q..E...K.NST..K..ISSTI...Q...RT.AKA.PR..AF.....GE..T.T...S...T.AQN...TGSAN..A....M
CAN34702    .GI.N.LS...RS..Q..E...K.NST..K..ISSTI...Q...RT.AKA.PR..AF.....GE..T.T...S...T.AQN...TGSAN..A....M
CAN34802    .GI.N.LS...RS..Q..E...K.NST..K..ISSTI...Q...RT.AKA.PR..AF.....GEK.T.T...S...T.AQN...TGSAN..A....M
CAN19702    .G..N.LS.A.RS..Q..E...K.NST..K..ISSTI...Q...RT.AKA.PR..A.....GE..T.T...S...T.AQN...TGSAN..A....M
JMLEIC72    .GI.N.LS...RS..Q..E...K.NRT..K..ISSTV...Q...RT.AKV.PRA.AP.....GE..T.TP.....T..QN...TGSAN..A....MQN.HTNIARPN
JMLEIC2564  .GI.N.LS...RS..Q..E...K.NRT..K..ISSTV...Q...RT.AKV.PRA.A.....GE..T.TP.....T..QN...TGSAN..A....MQN.HTNIARPN
JMRCGP558   .GI.N.LSS..RS..Q..G...KIN.T..K..ISSTV...Q..TRT.AKAATRA.A..M...GE..T.T...S...T..QN...TGSAN..A....MQK
JMLEIC506   .GI.N.LSS..RS..Q..K...K.T.T..KR.I.STV...Q...RT.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN..A..N.M
JMLEIC399   .GI.N.LSS..RS..Q..K...K.T.T..KR.I.STV...Q...RT.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN..A..N.M
JMLEIC1294  .GI.N.LSS..RS..Q..K...K.T.T..KR.I.STV...Q...RT.AKA.PRA.AF.....GE..T.....SN..T..QN...TGSAN..A..N.M
JPS03240    .GI.N.LSS..RS..Q..K.K.N.T.T..KR.I.STV...Q...RT.AKA.PRA.A.....GE..T.T...SN..T..QN...TGSAN..A..N.MQN
JPS03187    .SI.N.LSS..RS..Q..K...N.T.T..KR.I.STV..KQ...RT.AKA.PRA..F.....GE..T.T...SN..T..QN...TGSAN..A..N.MQN
hMPV172000  ....N.LSS..RS.AQ..E...K.KST...R...STA.S.Q...R...KAI.RA..F.M...E...T.TL..Y...T..QN...TGSAN..A.V..M
hMPV162000  ....N.LSS..RS.AQ..E...K.KST...R...STA.S.Q...R...KAI.RA..F.M...E...T.TL..Y...T..QN...TGSAN..A....MQN.HTNNIKPN
NL302       ....N.LSS..RS.VQ..EN..K.KLT...R..LSTA.S.Q...R...KAI.RA....M...GR..T.TL..S...T..QN...TGSAN..A....MQN.HTNNIKPN
CAN16402    ....N.LSS..RS.AQ..EN..K.N.TA..R...STA.S.Q..QR.A.KAI.RA....M...GR..T.T...S...T..QN...TGSAN..A.V..MQN
CAN9783     ....N.LSS..RS.AQ..E...K.K.T...R...STA.S.Q...R...KAI.RA..F.M...G...T.T...S...T..QN...TGSAN..A....MQN
NL1700A2    ....N.LSS..RS.AQ..E...K.K.T...I...NTA.S.Q...RT..KAI.RA..F.M...G...T.TL..S...T..QN.G.TGSAN.RA..G.VQD
CAN22702    ....N.LSS..RS.AQ..E...K.NLT...S...STA.S.Q.Q.R...KAIPRA.AF.M...G...T.TL..S...T..QN.G.TGSAN.RA..G.VQD
CAN20802    ....N.LSS..RS.AQ..E...K.NLT...S...STA.S.Q.Q.R...KAIPRA.AF.M...G...T.TL..S...T..QN.G.TGSAN.RA..G.VQD
CAN21502    ....N.LSS..RS.AQ..E...K.NLT...S...STA.S.Q.Q.R...KAIPRA.VF.M...G...T.TL..S...T..QN.G.TGSAN.RA..G.VQD
CAN18202    ....N.LSS..RS.AQ..E...K.NLT...S...STA.S.Q.Q.R...KAIPRA.VF.M...G...T.TL..S...T..QN.G.TGSAN.RA..G.VQD
NL396       S...N.LSS..RS..Q..E..A..K.T..K..I.STV...Q..LR...KA.LRA.AF.M...GEG.T.T...S...T..QN...TGSAN..A....MQN.HTNIAKPN
NL393       ....N.LSS..RS..Q..E...K.KLT..K..I.STV...Q.SIR...KA.LRA.AF.....GE..T.T...S...T..QN...TGSAN..A....MQN
NL181       ....N.LSS..RS..Q..E...K.K.T.......STV...Q..LR...KA.LRA.AF.........T.T...S...T..QN...T.SSN..A....MQD.DTNNTKQN
NL193       ....N.LSSA.RS..Q..E...K.KLT......LSTA...Q...R...KA.LRD.AFH....G...T.T...SG..T..QN...T.SSN..A....MQD
JMLEIC1011  .G..N..S...RS..Q..E...K.NST..K.KTSQVQFLE...HHGQQ.RRSPEPPPFARAAQEKDQPQHQSSLTT.PQP.IMKKQV
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